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Factors regulating arteriolar tone during microvascular growth

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Factors Regulating Arteriolar Tone during Microvascular Growth

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ABSTRACT

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Growth of the arteriolar network is accompanied by progressive changes in pressure and flow, and in the metabolic environment to which the arterioles are exposed. This dissertation was carried out to investigate the extent to which mechanisms involved in local blood flow regulation may also change during this growth. We isolated gracilis muscle arterioles from weanling (age 25-26 days) and juvenile (44-66 days) rats, and studied the responsiveness of these vessels to both myogenic and endothelium-dependent stimuli. Overall arteriolar responses to the endothelium-dependent agonists acetylcholine (ACh), A23187, VEGF and simvastatin were not different between age groups. Responses of juvenile arterioles to ACh and simvastatin were significantly reduced by inhibition of nitric oxide synthase or cyclooxygenase, by the hydrogen peroxide scavenger catalase, and by potassium channel inhibition. Responses of weanling arterioles to ACh and simvastatin were unaffected by these treatments, but significantly reduced by heme oxygenase inhibition. Arteriolar growth during juvenile maturation is accompanied by an increase in myogenic responsiveness, possibly because endothelium-derived PGH_2 or TXA_2 assumes a role in reinforcing myogenic activity over this period. In conclusion, mediators of endothelium-dependent dilation change during growth, with carbon monoxide contributing largely to these responses in young animals, and a combination of nitric oxide, prostanoids and hydrogen peroxide contributing to these responses in older animals. These data suggest that age-dependent differences in the control of vascular tone exist and may have significant implications for the regulation of tissue perfusion.

DEDICATION

I would like to dedicate this work to my parents, Pamela and Patrick Balch, who have offered unwavering encouragement and support, instilled in me the self-confidence to fulfill my dreams, and who have stood by my side through every decision, accomplishment, and failure. I love you both so much. Thanks for being the amazing parents you are.

PREFACE

This dissertation will begin with a review of the literature. The following four studies will be reported in manuscript form, followed by a general discussion and conclusion of the findings.

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GLOSSARY OF ABBREVIATIONS

α_2	Alpha-2
AA	Arachidonic acid (Arachidonate)
AC	Adenylyl cyclase
ACh	Acetylcholine
ADP	Adenosine diphosphate
δ-ALA	δ-Aminolevulinic acid
Ang II	Angiotensin II
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BK	Large-conductance calcium activated potassium channels
Ca^{2+}	Calcium
$[Ca^{2+}]_i$	Intracellular calcium concentration
$[Ca^{2+}]_o$	Extracellular calcium concentration
$CaCl_2$	Calcium chloride
CaM	Calmodulin
cGMP	3',5'-cyclic guanosine monophosphate
CO	Carbon Monoxide
CO_2	Carbon Dioxide
COX	Cyclooxygenase
CP450	Cytochrome P450
CrMP	Chromium (III) mesoporphyrin IX chloride
CuZnSOD	Copper zinc superoxide dismutase

DAG	Diacylglycerol
DCF	2’7’-dichlorofluoroscein
DCFH-DA	2’7’-dichlorodihydrofluoroscein diacetate
DiHETE	Diydroxyeicosatrienoic acid
D_{max}	Maximum diameter
D_{SS}	Steady-state diameter
DMSO	Dimethylsulfoxide
EC	Endothelial cell
ecSOD	Extracellular superoxide dismutase
EDCF	Endothelium-derived contracting factor
EDHF	Endothelium-derived hyperpolarizing factor
EDRF	Endothelium-derived relaxing factor
EDTA	Disodium ethylenediamine tetraacetate
EET	Epoxyeicosatrienoic acid
eNOS	Endothelial nitric oxide synthase (also NOS3)
ET	Endothelin
ETOH	Ethyl alcohol
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
Glib	Glibenclamide
GSH-PX	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
H_4B	Tetrahydrobiopterin

20-HETE	20- hydroxyeicosatrienoic acid
HLL	Heme-L-lysinate
HO-1	Heme oxygenase 1 (inducible form)
HO-2	Heme oxygenase 2 (constitutive form)
HO-3	Heme oxygenase 3 (constitutive form)
HRP	Horse radish peroxidase
Hsp	Heat shock protein
Ibtx	Iberiotoxin
iNOS	Inducible nitric oxide synthase (also NOS2)
i.p.	Intraperitoneal
IP_3	Inositol 1,4,5-trisphosphate
i.v.	Intravenous
K^+	Potassium
K_{Ca}	Calcium-activated potassium channels
KCl	Potassium chloride
K_{ATP}	ATP-sensitive potassium channels
L-NAME	N^{ω}-nitro-L-arginine methyl ester
L-NIO	N-iminoethyl-L-ornithine
L-NMMA	N^G- monomethyl-L-arginine
LOX	Lipoxygenase
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
$MgSO_4$	Magnesium sulfate

MI	Myogenic index
mmHg	Millimeters of mercury
MnSOD	Mitochondrial manganese superoxide dismutase
MS	Mechanosensitive
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaH_2PO_4	Sodium phosphate monobasic anhydrous
$NaHCO_3$	Sodium hydrogensulfite
NaOH	Sodium hydroxide
NE	Norepinephrine
NH_3	Ammonia
NO	Nitric oxide
nNOS	Neuronal nitric oxide synthase (also NOS1)
NOS	Nitric oxide synthase
NS 1619	1-(2'-hydroxy-5'-trifluoromethylphenyl)-5-trifluoromethyl-2(3*H*)benzimidazolone
O_2	Oxygen
$O_2^{\cdot-}$	Superoxide radical
$OH^{\cdot-}$	Hydroxyl radical
$ONOO^{\cdot-}$	Peroxynitrite
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PE	Polyethylene
PGE_2	Prostaglandin E_2 (Dinoprostone)

PGH_2	Prostaglandin H_2
PGI_2	Prostaglandin I_2 (Prostacyclin)
PI_3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKG	Protein kinase G
PPOH	6-(2 proparglyoxylphenyl) hexanoic acid
PSS	Physiological Saline Solution
ROS	Reactive oxygen species
Ser	Serine
SE	Standard error
sGC	Soluble guanylyl cyclase
SHR	Spontaneously hypertensive rats
Sim	Simvastatin
SMC	Smooth muscle cell
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
TEA	Tetraethylammonium
TXA_2	Thromboxane A_2
VEGF	Vascular endothelial growth factor
VGC	Voltage gated calcium channels
VSM	Vascular smooth muscle
VSMC	Vascular smooth muscle cell
WKY	Wistar Kyoto rat
XD	Xanthine dehydrogenase

XO	Xanthine oxidase

I. LITERATURE REVIEW

1. Introduction

The cardiovascular system is comprised of the heart, blood vessels, and blood. As early as the 2nd Century AD, Greek physician Galen of Pergamum studied this complex circulatory system and proposed that blood was made in the liver, pumped by arterial pulsations, passed from one side of the heart to the other via invisible pores in the dividing membrane, and was delivered to the organs of the body where it was consumed, rather than recycled to the heart. He also strongly argued that the venous and arterial systems were separate. Galen's teachings remained relatively unchallenged for centuries. It was not until 1628 that English physician William Harvey correctly described how blood was pumped around the body by the heart before returning to be re-circulated in a closed system (Harvey, 1628).

The cardiovascular system is responsible for transporting nutrients, water, electrolytes, hormones and oxygen (O_2) to organs and cells within the body, and eliminating products of metabolism, such as carbon dioxide (CO_2) and ammonia (NH_3). It not only functions as a transport system, but also contributes to homeostasis by maintaining body temperature, regulating body pH, increasing blood flow to meet energy demands, transmitting antibodies and white blood cells to sites of infection, and mediating hemostasis (Sherwood, 2007).

The large elastic and muscular arteries transport blood from the heart to the organs and serve as a pressure reservoir. The muscular arterioles are the major resistance vessels in the vascular tree and determine, by changes in vessel caliber, the amount of

blood that flows to a given tissue (Bevan, 1987; Segal, 2000; Hester & Hammer, 2002). Although multiple factors influence microvascular tone, such as circulating hormones, physical forces, sympathetic input, and local chemical changes, this dissertation will focus on vascular smooth muscle (VSM) and endothelial cell function in skeletal muscle arterioles. The following literature review will highlight the fundamental importance of endothelium-derived autacoids, myogenic activity, reactive oxygen species (ROS), and carbon monoxide (CO) to vascular function. The experimental approaches taken to study each of these areas will also be explored. It will conclude with a discussion on how vascular function changes with postnatal growth and maturation.

2. Mechanisms of Vascular Control

A. Endothelium-Dependent Control of Vascular Tone

1. General Aspects

The vascular endothelium, which lines the lumenal surface of all vessels, lies between the circulating blood and the VSM. Only a few decades ago, the endothelium was considered to have few functional properties. It is now well documented that the endothelium is a regionally specific, multifunctional organ that plays a pivotal role in maintaining cardiovascular homeostasis, and that can also contribute to the pathological mechanisms of numerous cardiovascular diseases (Rubanyi, 1993). The multiple duties performed by the endothelium include endocrine, exocrine, cell adhesion, clotting, growth, remodeling, and transport functions, provision of a physical permeability barrier, mediation of inflammatory responses, modulation of VSM tone, and adaptation to intermittent hypoxia (Lew *et al.*, 1989; Pohl & Busse, 1990; Manukhina *et al.*, 2006;

Pries & Kuebler, 2006; Sherwood, 2007). In 1980, Furchgott and Zawadzki were the first to report their in vitro observations that vasodilation to some agonists is dependent on an intact and healthy endothelium (Furchgott & Zawadzki, 1980). Consequently, endothelial dysfunction can lead to vasospasm, thrombosis, atherosclerosis, restenosis, and an impaired ability to reduce vascular resistance (Rubanyi, 1993; Rongen *et al.*, 1994).

The vascular tree consists of arteries, arterioles, capillaries, venules and veins, all of which are lined with a thin layer of smooth, flat endothelial cells. Conduit arteries differ from resistance arteries in size and function, as well as local environment (Agewall *et al.*, 2006). Whereas arteries are thick, highly elastic and have large radii, arterioles are highly muscular, and have well-innervated walls and small radii (Sherwood, 2007). Furthermore, Joannides and colleagues (2006) demonstrated a great heterogeneity of mechanisms and alterations in vasomotor responses between conduit and resistance arteries in various disease states suggesting the presence of different pathophysiological processes and degrees of alteration in endothelial function. Others have established that even though endothelial dysfunction occurs at the level of both conduit and resistance arteries in hypertension and coronary artery disease, the kinetics of these alterations differs (Anderson *et al.*, 1995; Neunteufl *et al.*, 2000; Park *et al.*, 2001). For example, alteration in endothelial function occurs earlier in resistance arteries than in conduit arteries in atherosclerosis (Joannides *et al.*, 2006), and acetylcholine induces less vasodilation in resistance arteries and constriction in conduit arteries in hypertension (Zeiher *et al.*, 1991; Houghton *et al.*, 1998; Belhassen *et al.*, 2001).

One of the ways in which the endothelium regulates cardiovascular homeostasis is through the release of multiple factors that influence permeability and vascular tone (Kubes, 1995). The three main vasodilators which are released by the endothelium are endothelium-derived relaxing factor (EDRF, also known as nitric oxide (NO)) (Furchgott & Vanhoutte, 1989), prostacyclin (PGI_2) (Moncada *et al.*, 1976), and endothelium-derived hyperpolarizing factor (EDHF) (Chen *et al.*, 1988; Feletou & Vanhoutte, 1988). These mediators can function independently or can operate together in a complex, but integrated manner to regulate vascular tone (Villar *et al.*, 2006). For example, PGI_2 can facilitate NO release from endothelial cells (ECs) and NO can activate PGI_2 synthesis (Davidge *et al.*, 1995). However, there is no uniform contribution of these dilators between vascular beds or species. Sometimes the contribution of PGI_2 and EDHF to endothelium-dependent relaxation is only evident after NOS inhibition or in the absence of NO (Feletou & Vanhoutte, 1996; Beverelli *et al.*, 1997; Wu *et al.*, 2001). Although NO appears to be the predominant vasodilator in some conduit vessels (Villar *et al.*, 2006), investigators have demonstrated that as the size of the vessel diminishes, the contribution that EDHF makes to endothelium-dependent dilation increases (Shimokawa et al, 1996).

Several other endothelium-derived vasoactive factors can be released, including adenosine triphosphate (ATP), adenosine diphosphate (ADP), serotonin, substance P, various cytochrome P450 (CYP) metabolites, adenosine, and a host of endothelium-derived contracting factors (EDCFs) that include endothelin (ET) and various arachidonic acid (AA) metabolites such as Prostaglandin H_2 (PGH_2) and thromboxane A_2 (TxA_2) (Furchgott & Zawadzki, 1980; Furchgott & Vanhoutte, 1989; Marshall & Kontos, 1990;

Dai *et al.*, 1992; Huang & Koller, 1997). These multiple endothelium-derived vasoactive factors can be released in response to numerous stimuli, including hemodynamic shear stress, decreases in perivascular PO_2, angiotensin II (Ang II), vasopressin, thrombin, cytokines, autonomic and sensory stimulation, hormones, thrombin, temperature changes, vascular endothelial growth factor (VEGF), and increases in metabolic demand (Rubanyi *et al.*, 1986; Pohl & Busse, 1989; Vanhoutte, 1989; La & Reid, 1995).

2. Nitric Oxide

Nitric Oxide (NO), originally named Endothelium-Derived Relaxing Factor (EDRF) before its chemical nature was determined (Furchgott & Zawadzki, 1980), was the first gas to be identified as an endogenously generated cell signaling and effector molecule (Ignarro *et al.*, 1987). NO regulates blood flow, affects glucose uptake into muscle fibers, and inhibits glycolysis, mitochondrial respiration, creatine kinase and muscle contraction (Reid, 1998).

NO is produced by conversion of L-Arginine to L-Citrulline via a five-electron oxidation by the enzyme nitric oxide synthase (NOS) (Palmer *et al.*, 1988). This reaction requires three substrates: L-arginine, O_2, and nicotinamide adenine dinucleotide phosphate (NADPH), and four cofactors: tetrahydrobiopterin (H_4B), calmodulin (CaM), flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN) (Sessa *et al.*, 1992; Griffith & Stuehr, 1995).

NO initiates relaxation of the VSM by activating soluble guanylyl cyclase (sGC) (Hobbs, 1997), which causes a rise in cyclic guanosine 3', 5'-monophosphate (cGMP) (Furchgott *et al.*, 1984). cGMP acts as a second messenger in a variety of physiological

processes, including vasodilation and neuronal signal transduction (Collier & Vallance, 1989). cGMP can activate cGMP-dependent protein kinase G (PKG), phosphorylate heat shock protein (Hsp) 20 (Brophy *et al.*, 2002), and can hyperpolarize the smooth muscle by opening calcium-activated potassium (K_{Ca}) channels (Carrier *et al.*, 1997). This in turn leads to inhibition of voltage-gated Ca^{2+} (VGC) channels and decreased entry of Ca^{2+} into cells. In smooth muscle, the drop in intracellular calcium $[Ca^{2+}]_i$ leads to relaxation (Carvajal *et al.*, 2000).

There are three major isoforms of nitric oxide synthase: NOS1 (neuronal, nNOS), NOS2 (inducible, iNOS), and NOS3 (endothelial, eNOS) (Forstermann *et al.*, 1998). eNOS and nNOS are considered constitutive, meaning they are present and active under normal, steady-state conditions. The NOS enzymes are dimers consisting of two identical myristoylated and palmitoylated subunits; only the dimer is able to bind substrate and cofactor (Fleming & Busse, 1999). The eNOS enzyme is controlled both via transcriptional and post-translational regulation. Post-translational regulation includes Ca^{2+}/CaM binding, fatty acid modification (myristoylation/palmitoylation), alterations in intracellular translocation, substrate and cofactor bioavailability, dimerization, binding to cofactors, and phosphorylation (Musicki & Burnett, 2006).

There are at least two distinct signaling pathways for eNOS activation in the endothelium: Ca^{2+}-dependent and Ca^{2+}-independent activation (Fleming & Busse, 1999; Ungvari *et al.*, 2001; Wyatt *et al.*, 2004). Receptor-dependent agonists such as ACh, adenosine, ATP, and bradykinin (BK) (Busse & Mulsch, 1990; Korenaga *et al.*, 1993; Muller *et al.*, 1999) and receptor-independent agonists such as calcium ionophores (Busse *et al.*, 1993; Huang & Koller, 1996) stimulate eNOS in a Ca^{2+}-dependent fashion.

Activation of NOS coupled with Ca^{2+} release is transient and produces NO rapidly (Bredt & Snyder, 1990). Although eNOS is the most sensitive NOS isoform to changes in intracellular Ca^{2+} levels (Luckhoff *et al.*, 1988), it can also be activated by fluid shear stress without a sustained increase in $[Ca^{2+}]_i$ (Ayajiki *et al.*, 1996; Fisslthaler *et al.*, 2000). This Ca^{2+}-independent pathway involves activation of phosphatidylinositol 3-kinase (PI_3K) and serine (Ser) kinases Akt and protein kinase A (PKA), which cause phosphorylation of Ser^{1177} on eNOS, thereby increasing eNOS activity (Dimmeler *et al.*, 1999).

3. Endothelium-Derived Arachidonic Acid Products

Arachidonic acid, a polyunsaturated essential fatty acid that is cleaved from membrane phospholipids by phospholipases, is metabolized to eicosanoids by the cyclooxygenase (COX), lipoxygenase (LOX), or CYP monooxygenase pathways (Imig, 1999). The COX enzyme catalyzes the rate-limiting step in prostaglandin synthesis by converting AA to into prostaglandin H_2, which can then be further metabolized to PGI_2 or dinoprostone (PGE_2) (Funk, 2001), both of which can relax VSM by stimulating either guanylyl cyclase (GC) or adenylyl cyclase (AC) (Gryglewski *et al.*, 1988; Waldman & Murad, 1988). PGI_2 elicits its powerful vasodilation by stimulating a cell-surface receptor (IPR) which leads to increasing levels of cyclic adenosine 3',5'-monophosphate (cAMP) and sequestration of Ca^{2+} (Vanhoutte & Scott-Burden, 1994; Tanaka *et al.*, 2004). Whereas the primary products of LOX are multiple forms of leukotrienes (LTs) (Funk, 2001), the CYP enzymes form epoxyeicosatrienoic acids (EETs), dihydroxyeicosatrienoic acids (DiHETEs), and 19- and 20-hydroxyeicosatetraenoic acids

(19- and 20-HETE, respectively) from AA (Roman *et al.*, 2000). These metabolites have been shown to play an essential role in regulating vascular function (Campbell & Harder, 1999; Roman *et al.*, 2000). For example, EETs can modulate intracellular Ca^{2+} levels in endothelial cells resulting in hyperpolarization (Rueben *et al,* 2004), and 11,12-EET activates protein kinase A (PKA) leading to afferent arteriolar vasodilatation (Imig *et al.*, 1999). Furthermore, whereas EETs dilate vessels by hyperpolarizing VSM cells through the activation of K_{Ca} channels (Roman *et al.*, 2000), 20-HETE exerts its constrictor effects via K_{Ca} channel inhibition and subsequent depolarization of the VSM (Zou *et al.*, 1996; Harder *et al.*, 1997). Inhibition of endogenous 20-HETE production prevents the vasoconstrictor response to elevations in local PO_2, suggesting that 20-HETE may serve as a vascular O_2 sensor (Harder et al, 1996; Lombard et al, 1999a). 20-HETE can also mediate myogenic responses, as inhibition of its production in renal and cerebral arterioles reduces pressure-dependent myogenic tone (Harder *et al.*, 1997; Roman *et al.*, 2000).

4. Endothelium-Derived Hyperpolarizing Factors

Not all endothelium-dependent relaxations can be fully explained by the release of either NO or vasodilator prostanoids (Feletou & Vanhoutte, 1996). There are various relaxing factors which are named EDHF because of their role in hyperpolarizing VSM (Bolz, de Wit & Pohl, 1999; Ungvari & Koller, 2001). Although several substances have been proposed, including cyclic AMP, c-type natriuretic peptide (CNP), metabolites of AA by CYP epoxygenase (such as EETs), hydrogen peroxide (H_2O_2), and potassium ion (K^+) (Feletou & Vanhoutte, 1996; Edwards *et al.*, 2000; Matoba *et al.*, 2000; Campbell &

Gauthier, 2002), only a few of these chemical factors have been conclusively determined to be EDHFs. Of the identified EDHFs, the particular molecule(s) that contribute to the regulation of vascular tone can differ depending on vascular bed and species (Feletou & Vanhoutte, 1996).

VSM hyperpolarization can also occur through the electrotonic spread of hyperpolarization from ECs to SMCs (Yamamoto et al., 1998; Emerson & Segal, 2000; Coleman et al., 2001; Sandow et al., 2002), perhaps via myoendothelial gap junctions (Edwards et al., 2000). Electrical coupling between SMCs and ECs was demonstrated in isolated vessels by simultaneously impaling one EC and one SMC with microelectrodes. At rest and after challenge with ACh, membrane potentials were identical, suggesting that electrical signals conducted along the endothelium can be directly communicated to the SMCs (Emerson & Segal, 2000). Still others have proposed that the rise in SMC Ca^{2+} generates a diffusion gradient that drives Ca^{2+} through myoendothelial cell junctions and into the endothelial cells, thereby initiating the synthesis of NO (Dora *et al.*, 1997).

5. Experimental Approaches for Studying Endothelium-Dependent Responses

There are numerous experimental means that have been developed to study endothelium-derived responses. Only those approaches relevant to this dissertation project are highlighted in this section. As mentioned earlier, the release of endothelium-derived vasoactive factors can be induced by numerous exogenously applied substances, including ACh, the calcium ionophore A23187, NE, ET, VEGF, simvastatin, as well as by physical forces, such as shear stress (Vanhoutte, 1989; La & Reid, 1995). ACh

activates eNOS by stimulating endothelial muscarinic receptors coupled to G-proteins, which then form inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), leading to an increase in $[Ca^{2+}]_i$. Ca^{2+} then binds with CaM, promoting NADPH-dependent electron flux (Busse & Mulsch, 1990). The calcium ionophore A-23187 promotes receptor-independent entry of Ca^{2+} into ECs, which activates eNOS, thereby releasing NO to dilate vessels (Huang & Koller, 1996).

Simvastatin and VEGF stimulate NO production independent of Ca^{2+}, via activation of the PI_3K/Akt pathway, leading to direct phosphorylation and increased activity of eNOS (Dimmeler *et al.*, 1999; Kureishi *et al.*, 2000; Shiojima & Walsh, 2002). This Ca^{2+}-independent signaling pathway for eNOS activation can be inhibited by tyrosine kinase inhibitors such as erbstatin A (Ayajiki *et al.*, 1996), and the Hsp90-binding protein, geldanamycin (Fleming *et al.*, 1997; Ungvari *et al.*, 2001).

NO production from all NOS isoforms can be inhibited in a concentration-dependent manner by substituted L-Arginine analogs, such as N^G- monomethyl-L-arginine (L-NMMA), N-iminoethyl-L-ornithine (L-NIO), and Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) (Rees *et al.*, 1990). As described above, NO is produced by conversion of the required substrate L-Arginine to L-Citrulline via a five-electron oxidation by eNOS (Palmer *et al.*, 1988; Sessa *et al.*, 1992; Griffith & Stuehr, 1995). L-arginine, a positively charged guanidino group-containing basic amino acid at neutral pH which is involved in many physiological processes (Masic, 2006), must compete with the L-Arginine analogs for substrate binding (Moncada *et al.*, 1991). These analogs are reversible, as the inhibition can be overcome by exogenous addition of L-Arginine (Rees *et al.*, 1990). AA epoxygenation reactions can be selectively inhibited by

6-(2 proparglyoxylphenyl) hexanoic acid (PPOH), a synthetic acetylenic fatty acid (Wang *et al.*, 1998; Imig, 1999). PPOH inhibits the reaction of epoxide formation at arachidonate positions 11 and 12 by CYP4A2 and CYP4A3 isozymes, but does not inhibit the omega-hydroxylation reaction of CYP4A1 leading to 20-HETE formation (Wang *et al.*, 1998). To inhibit constrictor prostanoids, the prostaglandin H_2/thromboxane A_2 (PGH_2/TxA_2) receptor antagonist SQ-29548 can be utilized (Cseko, *et al.*, 2004).

The endothelium can also be removed to determine its role in mediating various vascular responses. There are generally four different means of de-endothelialization: mechanical (inserting a wire, hair, or pipette tip into the vascular lumen to abrade the endothelium), physical (infusing distilled water through a vessel), chemical (investigators can use either Triton X-100 or saponin to eliminate endothelial responses) and enzymatic (via trypsin) (Uluoglu & Zengil, 2003). With all of these methods, verification of endothelial elimination, either by functional or histologic assessment, must be performed.

B. Myogenic Control of Vascular Tone

Local regulation of blood flow, as demonstrated by phenomena such as active hyperemia and reactive hyperemia, can occur largely through adjustments in arteriolar tone that do not rely on a functioning endothelium. The myogenic response is an important example of such endothelium-independent behavior.

1. Historical Background and Definition

The myogenic "mechanism" or "response" was first described by Sir William Bayliss in 1902, when he noted that as pressure was raised inside the isolated dog carotid artery, "a powerful contraction took place in which the artery appeared to writhe like a worm…The reaction is…myogenic in nature" (Bayliss, 1902). Other investigators, at the time unable to replicate his findings, shunned his hypothesis, and developed their own explanations for this phenomenon (Hooker, 1911; Anrep, 1912). It was not until 1949 that the myogenic mechanism was accepted when Folkow demonstrated in several vascular beds a sustained vascular tone in the absence of neural input (Folkow, 1949).

The term myogenic response has traditionally referred to an alteration of vascular diameter in response to changes in intralumenal or transmural pressure (Johnson, 1964; D'Angelo & Meininger, 1994). Contraction by VSM occurs when stretch is applied to the muscle, and conversely, relaxation occurs following a reduction in stretch (Johnson, 1980; Su et al., 2003). Myogenic activity contributes to vascular resistance and assists in the maintenance of a relatively constant blood flow and capillary hydrostatic pressure (Davis & Hill, 1999). However, myogenic activity is only operational over a specific pressure range (Johnson, 1980; Falcone et al., 1993), and the magnitude of this activity varies depending on the vascular bed and branch-order of the vessel (Bouskela & Wiederhielm, 1979; Meininger et al., 1987).

2. Mechanisms

The mechanisms by which VSM cells transduce a mechanical change (wall deformation or stretch) into first an electrical signal, then a chemical response, and finally a mechanical outcome (diameter change) are not completely elucidated. However, most investigations converge on similar signaling pathways (See Figure 1 below). An increase in transmural pressure (i.e. a rise in the pressure gradient across the vascular wall) causes the vessel wall to stretch, increasing the activity of stretch-activated ion channels (SAC), also referred to as mechanosensitive (MS) channels, in the VSM membrane (Sachs & Morris, 1998). This increase in MS channel activity leads to a graded membrane depolarization, due to increased movement of sodium (Na^+) and/or Ca^{2+} into the cell, and a secondary influx of Ca^{2+} through VGC channels (Davis *et al.*, 1992).

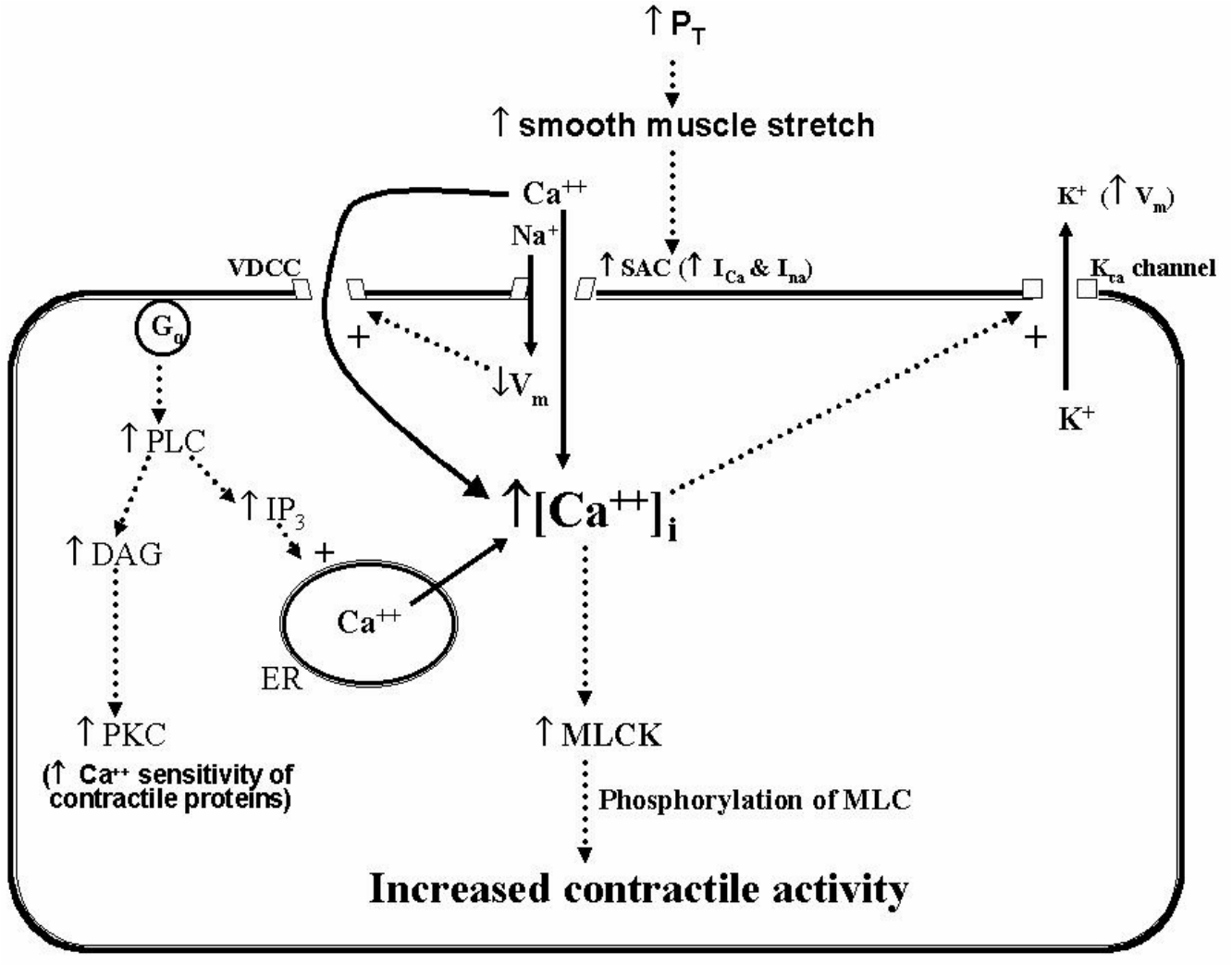
The myogenic mechanism is dependent in large part on the presence of extracellular Ca^{2+} ($[Ca^{2+}]_o$). For example, stretch-induced tone can be eliminated in resistance arteries in the presence of Ca^{2+} chelators (Hwa & Bevan, 1986a), or by incubating resistance arteries in a Ca^{2+}-free solution (Hwa & Bevan, 1986b). Furthermore, myogenic activation of VSM contraction and spontaneous action potentials of small renal arteries at higher transmural pressures can be blocked by Ca^{2+} channel inhibition with verapamil (Harder *et al.*, 1987). More recently, investigators have demonstrated a significant role for Ca^{2+} released from intracellular stores in modulating myogenic activity in the microvasculature (Zou *et al.*, 2000; Hill *et al.*, 2001).

The stretch-induced increase in $[Ca^{2+}]_i$ activates Ca^{2+}/CaM myosin light chain kinase (MLCK), which phosphorylates myosin ATPase, thereby increasing contractile activity (Horowitz et al., 1996). Increases in intralumenal pressure also stimulate membrane bound G proteins leading to the activation of phospholipase C (PLC), and subsequent formation of IP_3 and DAG (Narayanan *et al.*, 1994; Inscho *et al.*, 1998; see Figure 1). Whereas IP_3 leads to release of Ca^{2+} from the sarcoplasmic reticulum (SR), DAG activates the serine/threonine protein kinase C (PKC) (Osol et al., 1991; Narayanan et al., 1994). PKC activity is essential for myogenic Ca^{2+}-contraction coupling, as PKC inhibition completely abolishes myogenic activity (Wesselman *et al.*, 2001).

Several ancillary mechanisms have been proposed to mediate or sustain myogenic responses. Although K^+ channels do not initiate myogenic activity per se, Ca^{2+}-sensitive K^+ currents can modulate the intensity of myogenic responses (Brayden & Nelson, 1992; Nelson et al., 1995). Voltage-dependent K^+ (K_V) and Ca^{2+}-activated K^+ (K_{Ca}) channels can both repolarize membranes after VSM stretch (Davis & Hill, 1999). For example, inhibition of K_{Ca} channels augments myogenic tone in mesenteric arteries (Wesselman *et al.*, 1997). 20-HETE, a metabolite of AA, can sustain or initiate myogenic responses by inhibiting large-conductance K_{Ca} channels (Zou *et al.*, 1996; Harder *et al.*, 1997). Some investigators maintain that activation of smooth muscle-derived chloride channels (Yamazaki *et al.*, 1998) may also affect myogenic activity (Nelson *et al.*, 1997).

It appears as though initiation of the myogenic response may occur without endothelial input, as evidenced by maintenance of myogenic responsiveness after removal of the endothelium in many vessel types (McCarron *et al.*, 1989; Falcone *et al.*, 1991; Sun *et al.*, 1994; Wallis *et al.*, 1996). However, some studies have demonstrated

Figure 1: Smooth Muscle Pathways for Myogenic Activation



DAG: Diacylglycerol. PKC: Protein Kinase C. PLC: Phospholipase C. V_m: Membrane potential. VDCC: Voltage-dependent Ca^{2+} Channels. MLCK: myosin light chain kinase. IP_3: Inositol 1,4,5-trisphosphate. P_T: Transmural pressure. SAC: Stretch-activated channels.

increased myogenic responses with endothelial dysfunction (Huang *et al.*, 1993). Therefore, the endothelium clearly has the potential to influence the response due to release of one or more vasoactive factors (see Section 2A above). In fact, endothelium-derived NO has been shown to antagonize myogenic tone (Kuo *et al.*, 1991; van Breemen *et al.*, 1997; Nguyen *et al.*, 1999). \

Although controversial due to the method of endothelial removal, myogenic constriction in cat cerebral arteries was found to be completely dependent on the endothelium, as removal eliminated their ability to respond to changes in transmural pressure (Harder, 1987). Furthermore, other studies in either carotid (Rubanyi, 1988) or basilar (Katusic *et al.*, 1987) arteries have also found a role for the endothelium in pressure-dependent constriction, which may be a characteristic unique to the cerebral vasculature.

3. Experimental Approaches for Studying Myogenic Activity

Most of the earliest work investigating myogenic reactivity was obtained from in vivo experiments on whole organs (Bayliss, 1902; Folkow, 1949; Haddy *et al.*, 1957). However, in such complex integrative settings, flow-dependent mechanisms (Kuo *et al.*, 1990; Koller & Kaley, 1991) and metabolic responses (Hudlicka & el Khelly, 1985; Meininger *et al.*, 1987; Segal, 2005) may either enhance or minimize myogenic activity, thereby complicating interpretation of the results. Another in vivo technique, developed in the late 1970s, encloses the animal in a pressurized box with the vascular bed to be studied outside of the box (Bouskela & Wiederhielm, 1979). Pressure within the box is then either increased or decreased, and these pressure changes are transmitted equally to

both arterial and venous systems, thereby changing intravascular pressure without altering the pressure gradient for flow across the vascular bed of interest. A similar approach places only the tissue to be studied in a pressurized box. These pressurized box techniques are considered to be the best methods in which to study myogenic mechanisms in an in vivo setting.

More recently, in vitro approaches, such as wire-mounted rings/strips and isolated pressurized vessels, have become the principal approaches to study myogenic reactivity. Duling and colleagues (Duling et al., 1981) refined the isolated microvessel technique in the early 1980s, which allowed the myogenic response and its underlying mechanisms to be studied in more detail by eliminating potential metabolic, neural, humoral and endothelial influences. Experiments were first performed in small arteries (Osol & Halpern, 1985), and then in arterioles (Kuo *et al.*, 1988; Jackson & Duling, 1989), where the myogenic response is the most pronounced (Davis, 1993).

In isolated pressurized vessel protocols, segments of the vessel are cannulated (Laher & Bevan, 1993) and outflow occluded, such that changes in intralumenal pressure can be made without changes in flow (Halpern *et al.*, 1984). Alternatively, by connecting pressure and flow servo-control systems into the cannulation system, investigators can independently control both transmural pressure and intralumenal flow, such that constant flow can occur at preset pressure gradients (Laher & Bevan, 1993). These isolated vessels are subjected to a transmural pressure gradient similar to that experienced in the in vivo environment. Wire-mounted vascular strips are placed between two fine wires, one of which is attached to a fixed holder connected to a transducer, with the other attached to a micrometer (Mulvany & Halpern, 1976). The vessel is initially mounted

unstretched, allowed to equilibrate, and then stretched in an isometric fashion (Osol, 1991). Because the vessels are stretched circumferentially, they do not experience changes in transmural pressure (Osol, 1991). Some investigators (Johnson, 1980; Laher & Bevan, 1993) argue that isolated vessel strips or segments, which are isobaric preparations and are subjected to wall stretch in a similar fashion as experienced in vivo, may be the ideal preparations to study myogenic mechanisms.

C. Potassium Channels and Vascular Tone

1. General Aspects

Microvascular SMCs and ECs express several different types of ion channels which can determine and modulate membrane potential (Jackson, 2000, 2005), but for the purposes of this dissertation, the focus will be on K^+ channels. There are over 150 K^+ channel types, which can respond uniquely to various stimuli (Korn & Trapani, 2005). Whereas microvascular SMCs express inward-rectifier (K_{IR}), ATP-sensitive (K_{ATP}), voltage-gated (K_V) and large conductance Ca^{2+}-activated (BK_{Ca}) K^+ channels, microvascular ECs express K_{IR}, K_{ATP}, K_V, and small (SK_{Ca}) and intermediate (IK_{Ca}) conductance Ca^{2+}-activated K^+ channels (Jackson, 2005). However, it has been found that K_{Ca} channels are one of the major ion channels in VSM (Nelson & Quayle, 1995), and that BK_{Ca} channels are present in great quantities in arteriolar endothelial cells (Ungvari *et al.*, 2002).

K^+ channels play a pivotal role in controlling smooth muscle electrical and mechanical activities (Tanaka *et al.*, 2004) and are largely responsible for setting the vascular membrane potential (Fallet *et al.*, 2001; Korn & Trapani, 2005). Even a slight

change in the activity of K^+ channels is sufficient to alter membrane potential and tone (Quayle *et al.*, 1993; Nelson & Quayle, 1995). Currents through K^+ channels are always hyperpolarizing (Korn & Trapani, 2005), such that activation of K^+ channels leads to membrane hyperpolarization and relaxation, whereas inhibition leads to depolarization and contraction (Tanaka *et al.*, 2004; Ledoux *et al.*, 2006). As mentioned earlier, K^+ channels are generally thought to serve a negative feedback role in regulating myogenic constrictions (Nelson *et al.*, 1995; Knot & Nelson, 1998), whereas membrane depolarization plays a major role in the response of smooth muscle to stretch (Harder *et al.*, 1987; Knot & Nelson, 1995).

K^+ channels also play an instrumental role in communicating chemical signals generated in the endothelium to the VSM. For example, K^+ channels have been shown to play a role in the mechanisms by which NO, PGI_2, and EDHF exert their effects (Brayden, 2002; Tanaka *et al.*, 2004; Krummen *et al.*, 2005). BK_{Ca} channels in the VSM can be activated by EETs (Zhang *et al.*, 2001b). Endothelium-derived vasoconstrictors can close smooth muscle K_{ATP}, K_V, and BK_{Ca} channels through PKC, Rho kinase, or c-Src pathways, thereby leading to depolarization and the vasoconstrictor response (Jackson, 2005).

2. Pharmacological Approaches for Evaluation of Potassium Channel Activity

Because K^+ channels play such an important physiological role in regulating vascular membrane potential, investigators have used various tools to more fully understand K^+ channel pharmacology, structure, function, and mechanisms of activation.

Patch-clamp techniques are utilized to directly study K^+ channel activity and its influence on membrane potential in isolated cell membrane patches or single cells in vitro (Gebremedhin *et al.*, 1996; Prior *et al.*, 1998), whereas the development and discovery of pharmacological inhibitors and activators of K^+ channel activity have made it possible to test various hypotheses related to K^+ channel activity in vivo, albeit in a less direct fashion. Tetraethylammonium (TEA) can inhibit most K^+ channels (Korn & Trapani, 2005), but at relatively small concentrations it has been used to specifically block K_{Ca} channels (Nelson & Quayle, 1995). Iberiotoxin (Ibtx) and charybdotoxin (two scorpion venom peptides) are used for more specific inhibition of these channels (Galvez *et al.*, 1990; Gribkoff *et al.*, 1997). Scyllatoxin, biccuculline, dequalinium, clotrimazole, and UCL 1685 are inhibitors of various types of SK_{Ca} channels (Dunn, 1999; Strobaek *et al.*, 2000; Syme *et al.*, 2000), whereas the antidiabetic sulphonylureas glibenclamide and tolbutamide selectively block K_{ATP} channels (Standen *et al.*, 1989; Lombard *et al.*, 1999b; Brayden, 2002). Activators or openers of K^+ channels include the synthetic BK_{Ca} channel opener NS 1619 (1-(2'-hydroxy-5'-trifluoromethylphenyl)-5-trifluoromethyl-2(3*H*)benzimidazolone) (Olesen *et al.*, 1994; Fallet *et al.*, 2001). Chlorzoxazone, zoxazolamine, and riluzole have been used as SK_{Ca} channel stimulators (Syme *et al.*, 2000), whereas pinacidil, cromakalim, and diazoxide are K_{ATP} channel openers (Quayle *et al.*, 1997; Brayden, 2002; Mabanta *et al.*, 2006).

3. Reactive Oxygen Species and Vascular Tone

A. Background

ROS are formed via reduction of molecular O_2 during normal metabolic processes. With the generation of superoxide anion ($O_2^{\cdot-}$), other reactive intermediates can also be produced either spontaneously or enzymatically. The complete reduction of O_2 to H_2O requires several steps, and in the process can generate numerous byproducts (see Table 1 below). Some of these O_2-derived intermediates are chemically reactive due to unstable electron configurations, and can lead to the formation of other free radicals. These reactive molecules can cause lipid peroxidation, DNA damage and protein degradation, and are commonly implicated in the progression of Alzheimer's disease, cancer, atherosclerosis, hypertension, and diabetes (Cadenas, 1989; Mullarkey *et al.*, 1990; Nakazono *et al.*, 1991; Munzel *et al.*, 1995; Rajagopalan *et al.*, 1996; Oskarsson & Hofmeyer, 1997).

The protonation of $O_2^{\cdot-}$ results in the formation of perhydroxyl radical, which is highly reactive. $O_2^{\cdot-}$ can also act as a Bronsted base in aqueous solutions to form a hydroperoxyl radical, thereby forming H_2O_2 in acidic environments. H_2O_2 is also formed when $O_2^{\cdot-}$ is dismutated by superoxide dismutase (SOD) at a neutral or acidic pH. Hydroxyl radical ($OH^{\cdot}$), one of the most potent reactive oxidants, can be formed spontaneously by the reduction of H_2O_2, through the interaction of $O_2^{\cdot-}$ with H_2O_2, or through the interaction between H_2O_2 and reduced metal ions (e.g. copper, iron). This latter means of $OH^{\cdot}$ production occurs via the Fenton reaction (Aruoma & Halliwell, 1987; Aruoma *et al.*, 1991). $O_2^{\cdot-}$ can also react with NO to generate peroxynitrite ($ONOO^{\cdot-}$), which can lead to severe tissue injury (Li & Shah, 2004).

B. Sources of ROS in the Vasculature

Endothelial and VSM cells can generate $O_2^{\cdot-}$, H_2O_2, NO, $ONOO^{\cdot-}$, as well as other radicals (Droge, 2001; Cai *et al.*, 2003; Wassman *et al.*, 2004) via the NOS, COX, LOX, NADPH oxidase, or CYP monooxygenase enzymes (Katusic, 1996; Lassegue & Clempus, 2003; Ardanaz & Pagano, 2006). NADPH oxidases, which are arguably the best studied enzymes of ROS production in the vasculature (Lassegue & Clempus, 2003), serve as a principal source of $O_2^{\cdot-}$ (Rajagopalan *et al.*, 1996; Babior, 1999; Gorlach *et al.*, 2000; Griendling *et al.*, 2000b; Smith *et al.*, 2001). NADPH oxidase is a respiratory burst, multicomponent enzyme that is important in host defense. Most of the components of this enzyme are distributed in VSMCs, ECs, and adventitial fibroblasts (Pagano *et al.*, 1997; Griendling *et al.*, 2000b; Ardanaz & Pagano, 2006). The NADPH oxidase enzyme can be activated by hypoxia-reoxygenation, flow cessation, membrane depolarization, hormones and growth factors (Al-Mehdi *et al.*, 1998; Griendling & Harrison, 1999; Griendling *et al.*, 2000a; Sohn *et al.*, 2003). Endothelial NADPH oxidases continuously generate low levels of $O_2^{\cdot-}$, most of which is produced intracellularly (Li & Shah, 2004). CYP enzymes responsible for metabolizing AA (specifically CYP2 and CYP4A) are also implicated in ROS production (Fleming, 2001). In the absence of cofactors or substrate, eNOS may become uncoupled and reduce molecular O_2 rather than transfer electrons to L-arginine (Vasquez-Vivar *et al.*, 1998), thereby generating $O_2^{\cdot-}$. Cardiovascular diseases including hypercholesterolemia, atherosclerosis, hypertension and ischemia/reperfusion injury all demonstrate impaired endothelial function and increased ROS, as eNOS

Table 1: Various steps and by-products in the reduction of molecular oxygen. Bracketed items highlight potential mechanisms of reactions

$O_2 + e^- \rightarrow O_2^{\cdot -} + e^-$ (superoxide radical)

[lipoxygenase, COX, CP450, XO, NADPH oxidase, uncoupled eNOS, mitochondrial electron transport chain complexes I and III]

$O_2 + 2e^- \rightarrow H_2O_2$ (hydrogen peroxide)

[xanthine oxidase or glucose oxidase]

$O_2^{\cdot -} + e^- \rightarrow H_2O_2 + e^-$ (hydrogen peroxide)

[spontaneous or via superoxide dismutase]

$O_2^{\cdot -} + H_2O \rightarrow HO_2^{\cdot} + OH$ *(hydroperoxyl radical) in acidic environments*

$HO_2^{\cdot} + e^- + H \rightarrow H_2O_2$ *(hydrogen peroxide) in acidic environments*

$O_2^{\cdot -} + Fe^{3+} \rightarrow Fe^{2+} + O_2$

[via Haber-Weiss reaction]

$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {}^-OH + OH^{\cdot}$ (hydroxyl radical)

[via Fenton reaction]

$H_2O_2 + e^- \rightarrow {}^-OH + OH^{\cdot}$ (hydroxyl radical)

[spontaneous]

$H_2O_2 \rightarrow H_2O + O_2$

[spontaneous or via catalase or glutathione peroxidase]

uncoupling occurs during such pathologic conditions (Cosentino *et al.*, 1998; Xia *et al.*, 1998).

Xanthine oxidoreductase (XOR), highly expressed on the lumenal surface of the endothelium, is a metalloflavoprotein found as one of two functionally distinct forms: the constitutively expressed xanthine dehydrogenase (XD), and xanthine oxidase (XO), which is generated by posttranslational modification of XD (Li & Shah, 2004). Both XD and XO catalyze the oxidation of hypoxanthine and xanthine to urate, but because XO requires the reduction of O_2, it is the enzyme responsible for generating $O_2^{\cdot -}$ and H_2O_2 as byproducts (Houston *et al.*, 1999; Harrison, 2002). However, in certain situations, such as ischemia, XD can be converted to XO, thereby generating ROS (Harrison, 2004).

The electron transport chain reactions of the inner mitochondrial membrane generate ROS due to the constant production and escape of electrons (Cadenas & Davies, 2000). ROS production occurs principally at complex I (NADH dehydrogenase) and complex III (ubiquinone-cytochrome bc1). Monoamine oxidase in the outer mitochondrial membrane has been proposed as an additional source of ROS (Chen *et al.*, 2003). Although mitochondria do not normally contribute significantly to total vascular ROS production (Pagano *et al.*, 1995; Pagano *et al.*, 1997), this contribution may be enhanced during pathological conditions, such as hypoxia (Chandel *et al.*, 2000) and diabetes (Nishikawa *et al.*, 2000).

C. Endogenous Scavenger Systems that Regulate ROS Levels

"Aerobic organisms have evolved a multitude of ways to protect themselves from the deleterious aspects of an aerobic existence"
(Pryor *et al.*, 2006)

The effects of ROS are highly dependent on their local concentrations, which in turn are a function of not only the rate of their production, but also their rate of degradation or inactivation. In order to minimize the deleterious effects of ROS, the human body manufactures antioxidant enzymes, proteins, and low molecular weight scavengers (Gutteridge & Halliwell, 1999). Dietarily obtained antioxidants may also reduce oxidative stress, and have been promoted under the assertion that they may reduce rates and/or severity of coronary artery disease, stroke, dementias, and various cancers (Ames *et al.*, 1993), all of which have increased levels of oxidant damage (Finkel & Holbrook, 2000).

Nonenzymatic antioxidant molecules include uric acid, ubiquinone, ascorbic acid (vitamin C), α-tocopherol (vitamin E), and glutathione (GSH) (Suttorp *et al.*, 1986; Harris *et al.*, 1992; Droge, 2001; Dickinson & Forman, 2002). Whereas vitamins C and E are only obtained through the diet, the body can synthesize GSH and ubiquinone de novo (Andree *et al.*, 1999). GSH is the major cellular thiol antioxidant buffer, which serves as a substrate for glutathione peroxidase (gpx1) thereby eliminating lipid hydroperoxides and H_2O_2 (Dickinson & Forman, 2002). Ubiquinone and vitamin E, both lipid soluble antioxidants found in mitochondrial and other cellular membranes (Andree *et al.*, 1999), aid in the removal of lipophilic radicals (Gutteridge & Halliwell, 1999). Ceruloplasmin, a copper-containing protein, protects the body from extracellular oxidant stress (Gutteridge & Stocks, 1981).

Antioxidant enzymes include the family of SODs, catalase, the thioredoxin system, gpx1, and heme oxygenases (Suttorp *et al.*, 1986; Yamawaki *et al.*, 2003;

Wassman *et al.*, 2004; Szasz *et al.*, 2007). The SODs convert to $O_2^{\cdot-}$ to H_2O_2, which is then degraded to water by either catalase or gpx1. There are three main SODs, which are expressed throughout all of the vascular layers (Faraci & Didion, 2004; Szasz *et al.*, 2007). Nonetheless, each SOD has a primary area of localization and distinctive means of regulation. Cytosolic CuZnSOD, a homodimer with similar amino acid sequence homology with extracellular SOD (ecSOD), is the predominant SOD isoform in the endothelium (Cai & Harrison, 2000). Its activity and mRNA expression can be increased by shear stress (Tao *et al.*, 2007). Manganese superoxide dismutase (MnSOD) is a nucleus-encoded mitochondrial matrix protein (Bannister *et al.*, 1987) whose expression is induced by VEGF (Abid *et al.*, 2001) and thioredoxin (Wassman *et al.*, 2004). Ang II can increase vascular ecSOD expression, which involves both an increase in ecSOD transcription and stabilization of ecSOD mRNA (Fukai *et al.*, 1999). Although the glycosylated, tetrameric CuZnSOD (ecSOD) protects against extracelluar $O_2^{\cdot-}$ (Fridovich, 1995), generally very low levels of SODs or catalase enzymes exist in human extracellular fluid (Gutteridge & Halliwell, 1999).

Gpx1 is found in the plasma, cellular cytosol, and in mitochondria, and uses glutathione to reduce H_2O_2 and lipid peroxides to their respective alcohols (Ursini *et al.*, 1995). Catalase, a homotetrameric heme-containing enzyme, is found primarily in peroxisomes and catalyzes the conversion of H_2O_2 to water and O_2 (Gutteridge & Halliwell, 1999; Szasz *et al.*, 2007). Whereas gpx1 is thought to be more important as a scavenger of endogenous basal levels of H_2O_2 (Harlan *et al.*, 1984; Suttorp *et al.*, 1986), catalase is thought to be more important under conditions in which there are large quantities of H_2O_2 (Suttorp *et al.*, 1986). Thioredoxin reductase with thioredoxin and

NADPH, all of which are expressed in EC and VSM cells (Yamawaki *et al.*, 2003), function together to lower ROS levels. Heme oxygenase, found in endothelial and VSM cells (Christodoulides *et al.*, 1995; Marks *et al.*, 1997; Yet *et al.*, 1997; Motterlini *et al.*, 1998; Sammut *et al.*, 1998; Parfenova *et al.*, 2001), degrades free heme, which can be pro-oxigenic, and generates biliverdin and bilirubin, both of which have antioxidase properties (Perrella & Yet, 2003).

D. H_2O_2 as a Second Messenger and Regulator of Vascular Tone

Although researchers traditionally believed that ROS were simply by-products of normal oxidative metabolism or functioned solely in an antimicrobial capacity, we now know that some ROS can also serve as signaling molecules for a variety of functions, including the regulation of vascular tone. $O_2^{\cdot -}$ is a relatively membrane impermeable and unstable radical that is rapidly dismutated to H_2O_2, a more stable and membrane-permeable molecule. Therefore, many biological effects that have been attributed to $O_2^{\cdot -}$ may actually be mediated by H_2O_2. Similar to NO, H_2O_2 can freely diffuse between cells, and is capable of traversing a distance equal to several cell diameters before reacting with another molecule or being catabolized (Griendling & Harrison, 1999; Chen *et al.*, 2003).

Several studies support the role of H_2O_2 as a signaling molecule for changes in VSM tone (Burke-Wolin *et al.*, 1991; Yang *et al.*, 1998a; Fujimoto *et al.*, 2001; Yada *et al.*, 2003). Depending on the vascular bed, species, or local concentration, H_2O_2 can elicit either contractile or relaxant responses. H_2O_2 was first proposed as an EDHF by Matoba and colleagues (Matoba *et al.*, 2000), after they found that catalase inhibited

ACh-induced dilation and VSM hyperpolarization in mouse mesenteric arteries. There is also evidence to suggest that H_2O_2 plays an important role in the autoregulation of coronary blood flow (Yada *et al.*, 2003), and in flow-induced dilation of both human coronary arterioles and human mesenteric arteries (Miura *et al.*, 2003; Mabanta *et al.*, 2006). Others have demonstrated that H_2O_2 is an EDHF in the BK-induced dilation of pig pial arterioles (Lacza *et al.*, 2002). It appears as though there is a role for H_2O_2-induced dilation in pathologic states as well. For example, in spontaneously hypertensive rats (SHR), relaxation of aortas to the calcium ionophore A23187 relies on the presence of H_2O_2 (Cosentino *et al.*, 1998). In coronary arterioles from patients with coronary artery disease, H_2O_2 can also contribute to flow-induced dilation (Miura *et al.*, 2003).

H_2O_2 can elicit vasodilation through both endothelium-dependent and endothelium-independent mechanisms (Zembowicz *et al.*, 1993; Barlow & White, 1998; Yang *et al.*, 1998a; Thengchaisri & Kuo, 2003). H_2O_2 has also been shown to increase endothelial NOS expression and activity (Cai *et al.*, 2003), and there is extensive evidence from other studies to indicate that NO release is in fact the predominant endothelium-dependent mechanism through which H_2O_2 induces dilation (Zembowicz *et al.*, 1993; Yang *et al.*, 1998a; Cai *et al.*, 2002; Shimizu *et al.*, 2003; Cseko *et al.*, 2004; Gil-Longo & Gonzalez-Vazquez, 2005). H_2O_2-induced dilation in the coronary microcirculation is also mediated, in part, by the release of endothelial PGE_2 and subsequent activation of smooth muscle prostaglandin receptors (Thengchaisri & Kuo, 2003). However, H_2O_2 can also induce VSM hyperpolarization and relaxation in an endothelium-independent manner via activation of K^+ channels (Campbell *et al.*, 1996; Sobey *et al.*, 1997; Miura *et al.*, 1999; Matoba *et al.*, 2000; Thengchaisri & Kuo, 2003).

The main contributing type of K^+ channel appears to depend on the species and/or vascular bed; in some cases the mediators are K_{Ca} channels (Campbell *et al.*, 1996; Miura *et al.*, 1999; Iida & Katusic, 2000; Miura *et al.*, 2003; Thengchaisri & Kuo, 2003), and in other cases K_{ATP} channels (Wei *et al.*, 1996; Filipovic & Reeves, 1997; Lacza *et al.*, 2002).

H_2O_2 has also been found to induce contractile responses. For example, H_2O_2 causes constriction of the aorta, pulmonary artery and superior mesenteric artery of the rat (Jin & Rhoades, 1997; Rodriguez-Martinez *et al.*, 1998; Sotnikova, 1998; Yang *et al.*, 1998b; Pelaez *et al.*, 2000a; Gao & Lee, 2001), pig pulmonary arteries (Pelaez *et al.*, 2000b), and canine basilar arteries (Yang *et al.*, 1999). H_2O_2 can also increase the tone of KCl-preconstricted mouse mesenteric arteries (Lucchesi et al., 2005).

There have been multiple proposed mechanisms of H_2O_2-induced vasoconstriction. For example, in rat gracilis muscle arterioles, removal of the endothelium partly reduces constrictions to lower concentrations of H_2O_2, but these constrictions are completely abolished either by inhibition of prostaglandin synthesis or antagonism of PGH_2/TxA_2 receptors, suggesting that H_2O_2 must elicit its constrictor effect via endothelial and smooth muscle PGH_2/TxA_2 release (Cseko et al., 2004). Consistent with this finding is the observation that H_2O_2 activates cyclooxygenase to produce PGH_2, among other prostaglandins (Hemler *et al.*, 1979). Others have demonstrated that H_2O_2 triggers increases in VSM $[Ca^{2+}]_i$ by direct stimulation of VGC channels, mobilization of intracellular Ca^{2+} from the sarcoplasmic reticulum via ryanodine receptors, inhibition of Ca^{2+} ATPase, or stimulation of IP_3-induced Ca^{2+} release (Grover & Samson, 1988; Grover *et al.*, 1992; Horowitz *et al.*, 1996; Favero *et*

al., 1995; Tabet *et al*., 2004). Increased intracellular Ca^{2+} allows for activation of myosin light chain kinase (MLCK), and subsequent phosphorylation of 20-kDa myosin light chains (MLC20), thereby leading to constriction (Ardanaz & Pagano, 2006).

E. Experimental Approaches for Detecting H_2O_2 In Vivo

Although many approaches have been developed to detect and quantify H_2O_2 concentrations, none are ideal. Fluorescent probes are usually linked to horse radish peroxidase (HRP), such that in the presence of H_2O_2, hydrogen donors can be oxidized by HRP, causing a change in fluorescence intensity (Boveris, 1984; Tarpey & Fridovich, 2001). Although some probes, such as scopoletin (7-hydroxy-6-methoxy-coumarin, Boveris *et al.*, 1977), result in decreased intensity in the presence of H_2O_2, other previously non-fluorescent probes increase in fluorescence intensity with H_2O_2 contact; these types of probes include 2′,7′ dichlorodihydrofluorescein diacetate (DCFH-DA, Miura *et al.*, 2003; Silveira *et al.*, 2003), *p*-hydroxyphenylacetate (Hyslop & Sklar, 1984), homovanillic acid (3-methoxy-4-hydroxyphenylacetic acid, Ruch *et al.*, 1983), and *N*-acetyl-3,7-dihydroxyphenoxazine (Cohen *et al.*, 1996). Spectrophotometric assays including tetramethylbenzidine (Staniek & Nohl, 1999) and phenol red (Pick & Keisari, 1980) are other options which also work via H_2O_2-induced oxidation. There are many drawbacks to using assays which depend on oxidation reactions, as cellular peroxidases, peroxynitrite, hypochlorous acid, and even NO can also oxidize the target compound, leading to inaccurate estimations of H_2O_2 concentration (Tarpey & Fridovich, 2001; Silveira *et al.*, 2003). Nanoelectrodes, which allow for amperometric detection of H_2O_2

at concentrations as low as 10^{-7} M, function via Prussian Blue (PB), which is the most effective electrocatalyst for H_2O_2 reduction (Vreeke *et al.*, 1994; Karyakin *et al.*, 2004; Ricci & Palleschi, 2005). Although nanoelectrodes allow for more quantitative and sensitive measurements of H_2O_2 concentrations, their in vivo use is limited by sensor selectivity (ascorbate can also react with PB) and instability (Karyakin *et al.*, 2004).

In addition to the methodological limitations, in vivo concentrations of H_2O_2 have been difficult to measure due to the complex H_2O_2 catalytic and degradation processes that occur and because of the contribution from intricate scavenger systems. H_2O_2 can readily react with heme proteins and protein sulfhydryl groups (van der Vliet *et al.*, 1994; Halliwell *et al.*, 2000). Both nonenzymatic antioxidant molecules, including uric acid, Vitamin C, Vitamin A, and glutathione (Droge, 2001; Dickinson & Forman, 2002), and enzymatic systems, including SODs, catalase, the thioredoxin system, glutathione peroxidases, and heme oxygenases (Cai & Harrison, 2000; Faraci & Didion, 2004; Szasz *et al.*, 2007) also affect relative H_2O_2 concentrations.

Nonetheless, various investigators have attempted to quantify H_2O_2 production in the vasculature. Cosentino and colleagues (1998) used electrochemical sensors to detect H_2O_2 concentrations in aortas from Wistar Kyoto (WKY) rats and SHRs. When pretreated with SOD and stimulated with the calcium ionophore A23187, these concentrations reached ~10 nmol/L in WKYs and ~60 nmol/L in SHRs. Others have measured the extracellular H_2O_2 concentration surrounding stimulated human neutrophils (Test & Weiss, 1984). When neutrophils were stimulated with the croton oil derivative PMA, H_2O_2 levels reached ~60 μM. H_2O_2 levels as high as ~9 μM/L were detected in blood from patients with a history of myocardial infarction drawn after exercise (Deskur

et al., 1998). In genetic hypertensive patients, plasma H_2O_2 levels were measured via electrode and found to be ~3 μM/L (Lacy *et al.*, 1998).

4. Carbon Monoxide and Vascular Tone

A. Background

Since the 19th century, it has been known that the blood contains CO (Grehant, 1894; Coburn *et al.*, 1963). In 1949, Torgny Sjöstrand demonstrated that CO is produced in man, finding higher CO concentrations in expired air than in inspired air (Sjostrand, 1949). CO, a simple diatomic, colorless gas at room temperature and pressure (Pryor *et al.*, 2006), has primarily been considered a toxic molecule, capable of displacing O_2 from hemoglobin and causing tissue hypoxia (Weaver, 1999) as well as cardiovascular and neurological damage (Penney, 1990). In fact, as little as 0.4% of CO in the air can cause death in less than one hour (Otterbein & Choi, 2000).

CO is generated mainly through the catabolism of the ubiquitous metalloporphyrin ferric heme (FeIII) by heme oxygenase (HO) enzymes. However, minor sources of CO production include auto-oxidation of phenols, flavenoids, and halomethanes, photo-oxidation of organic compounds, and lipid peroxidation of membrane lipids (Rodgers *et al.*, 1994; Archakov *et al.*, 2002).

HO is the only known enzyme that generates three reaction products (Ryter & Otterbein, 2004): heme is oxidatively cleaved to produce CO, biliverdin-IXα, and ferrous iron (FeII) in equimolar quantities (Yoshida & Kikuchi, 1978). The HO system was originally thought to only regulate cellular heme homeostasis, with its products

considered waste materials (Maines, 1997). However, biliverdin has been demonstrated to have antioxidant activities and iron prooxidant capabilities (Ryter & Tyrrell, 2000), and CO has been found to play a role as a second messenger in various biological systems (Marks *et al.*, 1991).

Three HO isoforms have been characterized: HO-1 (also referred to as heat shock protein 32), HO-2, and HO-3 (McCoubrey *et al.*, 1997; Naik *et al.*, 2003). Whereas HO-1 was identified in the early 1970s (Maines & Kappas, 1974; Yoshida *et al.*, 1974), HO-2 was not identified until the mid-1980s (Maines *et al.*, 1986; Trakshel *et al.*, 1986), and HO-3, still not fully characterized, was first identified in the late 1990's (McCoubrey *et al.*, 1997). HO-1, the inducible form of heme oxygenase, can be stimulated by many different factors and conditions, including hypoxia, hypertension, endotoxic shock, and shear stress (Johnson *et al.*, 1996; Wagner *et al.*, 1997; Yet *et al.*, 1997; Jernigan *et al.*, 2001). HO-2 is constitutively expressed in most tissues and is generally not inducible (Maines, 1997). HO-3 is also expressed constitutively, but its heme degrading activity is low (McCoubrey *et al.*, 1997).

Both HO-1 and HO-2 have been demonstrated in endothelial cells (Marks *et al.*, 1997; Yet *et al.*, 1997; Sammut *et al.*, 1998; Parfenova *et al.*, 2001) and VSM cells (Christodoulides *et al.*, 1995; Yet *et al.*, 1997; Motterlini *et al.*, 1998). HO-1 can be readily induced in the heart and blood vessels, but HO-2 is the predominant enzyme expressed under normal conditions in the cardiovascular system (Ewing *et al.*, 1994).

HO-1 and HO-2 are very different enzymes, coming from different gene products and sharing little similarity in amino acid composition, structure, or gene organization (Muller *et al.*, 1987; Cruse & Maines, 1988; Keyse & Tyrrell, 1989). They also differ

with regard to cell type, tissue distribution, and regulation. However, they are similar in their mechanism of heme catalysis, substrate specificity, and cofactor requirements (Maines *et al.*, 1986; Trakshel *et al.*, 1986).

B. Mechanisms by which CO Can Influence Biological Processes

Because CO can bind to heme moieties, it can either increase or decrease the activity of many enzymes that contain a heme group (Roberts *et al.*, 2004). For example, CO has been shown to interact with hemoglobin, myoglobin, catalase, cytochrome c oxidase, NADPH oxidase, soluble guanylyl cyclase, NOS, and CYP enzymes (Stone & Marletta, 1994; Maines, 1997; Ryter *et al.*, 2002; Ryter & Otterbein, 2004; Ishikawa *et al.*, 2005; Kim *et al.*, 2006; Pryor *et al.*, 2006). However, outcomes differ depending on the specific interaction.

Although CO has both vasodilatory and vasoconstrictor properties, it is generally considered to be a vasodilator molecule (Johnson *et al.*, 1999). CO relaxes rabbit (Furchgott & Jothianandan, 1991) and rat (Lin & McGrath, 1988) aorta, dog coronary arteries (Furchgott & Jothianandan, 1991), rabbit pulmonary arteries (Steinhorn *et al.*, 1994), rat tail arteries (Wang *et al.*, 1997a), rat afferent arterioles (Thorup *et al.*, 1999) and pial arterioles of newborn pigs (Leffler *et al.*, 1999).

Low levels of CO can induce release of NO from a large intracellular pool (Thorup et al., 1999) and can directly activate sGC (Furchgott & Jothianandan, 1991; Schmidt, 1992), thereby causing vasorelaxation. CO generated by HO activity in the cardiovascular system appears to increase tissue cGMP levels (Ewing *et al.*, 1994).

Although sGC is involved in CO vasodilation in rabbit aorta, as seen by increases in tissue cGMP levels (Furchgott & Jothianandan, 1991; Hussain *et al.*, 1997), inhibitors of sGC only partially reduce the relaxant effects of CO in either rat tail artery (Wang *et al.*, 1997a) or lamb ductus arteriosus (Coceani *et al.*, 1996).

Others have demonstrated that CO plays no role in activating GC in rat aortic and pulmonary artery SMCs (Morita *et al.*, 1995). Some of the cGMP-independent dilations are attributed to mechanisms involving CYP enzymes (Coceani *et al.*, 1994) and K_{Ca} channels (Wang *et al.*, 1997b; Leffler *et al.*, 1999; Zhang *et al.*, 2001a; Jaggar *et al.*, 2002). Coceani and colleagues (1994) found that CO relaxes lamb ductus arteriosus via inhibition of a cytochrome P450-based mono-oxygenase reaction controlling the formation of ET (Coceani *et al.*, 1994). CO elicits smooth muscle hyperpolarization by activating large-conductance K_{Ca} channels (Xi *et al.*, 2004), or increasing the Ca^{2+} sensitivity of these channels (Jaggar *et al.*, 2002; Xi *et al.*, 2004). More specifically, CO increases the open state probability of K_{Ca} channels via modification of a histidine residue on the external membrane side of these channels (Wang & Wu, 1997). Furthermore, CO-induced dilation of newborn porcine pial arterioles is blocked by COX and NOS (Leffler *et al.*, 2001), suggesting that perhaps COX-derived prostaglandins and NO play a role in CO-mediated dilation of these vessels.

The relaxing action of CO in some arteries has been found to be endothelium *independent* (Lin & McGrath, 1988; Furchgott & Jothianandan, 1991; Hussain *et al.*, 1997). In small rat mesenteric arteries, whereas exogenously applied CO resulted in dilation in the absence of an intact endothelium, endothelial removal eliminated the vasodilator response to endogenously produced CO (Naik *et al.*, 2003). These results

suggest that although the endothelium may be responsible for CO production, CO-mediated vasodilation occurs via actions on the VSM in a similar fashion to NO.

Although several studies have documented the ability of CO to inhibit NO synthesis by blocking NOS (White & Marletta, 1992; Pufahl & Marletta, 1993; Matsuoka *et al.*, 1994), fewer studies have demonstrated a constrictor role for CO. However, CO was shown to induce concentration-dependent vasoconstriction in the intact vascular bed of the rat hindlimb (Marks *et al.*, 2003). Induction of HO-1 has been shown to attenuate muscarinic agonist-induced NO release (Thorup *et al.*, 1999) and vasorelaxation (Kaide *et al.*, 2002) in isolated renal arteries. Both exogenously applied and endogenously formed CO induce constriction of rat gracilis muscle arterioles pretreated with phenylephrine (Johnson & Johnson, 2003). This latter study suggests that CO might exert its vasoconstrictor properties by inhibiting NOS, because L-arginine treatment abolished CO-induced vasoconstriction (Johnson & Johnson, 2003).

In some pathological states, HO expression and CO formation are increased, possibly leading to increased inhibition of NOS (see Section 4E below). Salt-induced hypertension in Dahl salt-sensitive rats (DS) is accompanied by increased production of endogenous CO, and aortic and arteriolar HO-1 protein levels (Johnson *et al.*, 2003). As early as 1984, investigators found that CO enhances the development of salt-induced hypertension in DS rats, although they were unable at that time to elucidate the underlying mechanism (Shiotsuka *et al.*, 1984). In support of an inhibition of NOS by CO during salt-induced hypertension is the observation that NOS inhibition with L-NAME has no effect on arteriolar tone in hypertensive DS rats, even though it causes arteriolar constriction in normotensive DS rats (Boegehold, 1992). This is consistent

with the possibility that increased CO production in these pathologic conditions may be responsible for NOS inhibition. Further supporting this theory is the finding that skeletal muscle arterioles from Dahl-Rapp salt-sensitive hypertensive rats do not demonstrate endothelium-dependent dilation, but these responses can be completely restored by in vitro treatment with a HO inhibitor (Johnson *et al.*, 2003). Finally, in transgenic mice that overexpress VSM HO-1, vasodilatory responses to NO are suppressed, primarily by impairing activation of sGC, leading to elevation of arterial pressure (Imai *et al.*, 2001).

Still others have found no role for CO-mediated vascular responses. CO at concentrations ranging from 10^{-6} to 3 x 10^{-4} mol/L does not appear to have a significant effect on tone in rabbit or dog cerebral arteries (Brian *et al.*, 1994). Other investigators found that the diameters of isolated, pressurized and perfused rat middle cerebral arteries were not altered by 10^{-6} to 10^{-4} M CO (Andresen *et al.*, 2006). Although CO regulates vascular tone in many tissues, perhaps it is not an important mediator of vascular function in the adult cerebral circulation, even though it may influence vascular responses in cerebral vessels from young animals (Leffler *et al.*, 1999).

C. Regulation of CO Production

As the information in Section 4A clearly indicates, there is not a sole regulator of HO activity and subsequent CO production in vivo (Cary & Marletta, 2001). However, HO levels are developmentally regulated, at least in the central nervous system. Whereas the Purkinje neurons of the cerebellum demonstrate intense HO-2 immunoreactivity in adult rats, this is not seen in two-week-old rats (Maines *et al.*, 1996). Steroids appear to play a major role in regulating HO-2, as corticosterone treatment in postnatal rats results

in enhanced gene transcription in the promoter region of HO-2, in increased HO-2 transcription, and in elevated levels of HO activity and protein (Maines *et al.*, 1996; Maines, 1997).

Regulation of CO production by cerebral microvessels can include stimulation of HO-2 catalytic activity by tyrosine phosphorylation (Leffler *et al.*, 2003). In neurons, HO-2 activity is stimulated by casein kinase 2 (CK2)-catalyzed phosphorylation of serine-79 (Boehning *et al.*, 2003). Nonetheless, one definite prerequisite for HO activity is the presence of sufficient substrate, in the form of heme or the heme precursor aminolevulinate (Leffler *et al.*, 2003).

D. Experimental Approaches for Studying CO and Determining its In Vivo Concentrations

Although there are multiple ways to detect CO, including infrared absorption, calorimetry, electrochemical methods based on selective membranes, gas chromatography, and radioisotope counting (Morimoto *et al.*, 2001), in vivo measurements are difficult. Nonetheless, tissue contents of CO are reported to be 1-50 pmol/mg fresh weight (Vreman *et al.*, 2000), which others have calculated to be approximately 1-50 μmol/l (10^{-6} – $5x10^{-5}$ M) (Johnson & Johnson, 2003).

Different experimental methods have been used to stimulate endogenous production of CO. The rate-limiting step in heme synthesis is the production in mitochondria of δ-aminolevulinic acid (δ-ALA) from succinyl coA and glycine, a reaction that is catalyzed by the tightly regulated enzyme δ-ALA synthase (Kikuchi & Hayashi, 1981; May *et al.*, 1990). Whereas heme-L-lysinate (HLL), another HO

substrate, is utilized in some experiments, δ-ALA is the preferred choice in others because HLL contains iron and may lead to iron loading and detrimental effects on tissues (Johnson & Johnson, 2003). However, the dose-dependent vasodilator response of HLL is mediated by a product of the HO reaction (Naik *et al.*, 2003).

Different metalloporphyrins are used to inhibit HO activity, including zinc protoporphyrin IX (ZnPPIX), tin protoporphyrin IX, chromium mesoporphyrin IX (CrMP) (Vreman *et al.*, 1993), zinc deuteroporphyrin IX 2,4-bis-ethylene glycol (ZnBG) (Chernick *et al.*, 1989), and zinc N-methylprotoporphyrin IX (ZnPP) (De Matteis *et al.*, 1985). The potency of metalloporphyrins in inhibiting HO is affected by the metal cation associated with the porphyrin ring and by different ring substituents (Vreman *et al.*, 1993), and therefore some chemicals appear to be better suited for experimentation purposes than others. Investigators have demonstrated some nonspecific effects of the metalloporphyrin HO inhibitors (Luo & Vincent, 1994; Appleton *et al.*, 1999). For example, metalloporphyrins have been suggested to either inhibit (Luo & Vincent, 1994) or activate (Chakder *et al.*, 1996) NOS, or to inhibit sGC (Grundemar & Ny, 1997). All of these reasons may help to explain the variability of vascular responses observed after HO inhibition (see below).

Some have found no effect of HO inhibition on rat tail artery (Wang *et al.*, 1997a), aorta (Kozma *et al.*, 1999) or femoral artery (Kozma *et al.*, 1999) diameters. Others have found that HO inhibition increases hindlimb resistance in animals pretreated with L-NAME, but not in those unexposed to L-NAME (Johnson *et al.*, 2002). Others have demonstrated that CrMP promotes constriction of gracilis muscle arterioles in the

presence of L-NAME (Kozma *et al.*, 1997; Kozma *et al.*, 1999), whereas CrMP has been found to dilate newborn pig pial arterioles (Leffler *et al.*, 1999).

Systemic administration of ZnPPIX increases renal vascular resistance, but does not change mean arterial pressure in Sprague-Dawley rats subjected to chronic hypoxia (Jernigan *et al.*, 2001). ZnPPIX does not affect either renal vascular resistance or mean arterial pressure in control rats (Jernigan *et al.*, 2001). In contrast, ZnPPIX administration leads to sustained increases in arterial pressure, vasoconstriction and elevated total peripheral resistance in Sprague-Dawley rats (Johnson *et al.*, 1995). These inconsistent findings may be due to the relative concentrations of ZnPPIX, as Johnson's group utilized 45 μmol/kg, whereas Jernigan's group utilized 11 μmol/kg. At higher concentrations, ZnPPIX can inhibit sGC and NOS (in addition to HO), which may account for the observed increases in mean arterial pressure and total peripheral resistance (Appleton *et al.*, 1999). Others have found that increased HO activity induced by tin chloride treatment is associated with a lowering of blood pressures of spontaneously hypertensive rats (Sacerdoti *et al.*, 1989).

E. CO vs. NO: Comparisons and Interactions

NO and CO are both simple gases with similar water solubilities that are produced endogenously by enzymatic means (Pryor *et al.*, 2006). They are equally capable of forming complexes with most hemoproteins at the heme iron center, and they can act as heme ligands (Maines, 1997). Both can activate sGC, induce vasorelaxation, and stimulate K^+ channel activity (Rees *et al.*, 1989; Furchgott & Jothianandan, 1991;

Bolotina *et al.*, 1994; Wang *et al.*, 1997a; Wang, 1998). NO and CO can also inhibit platelet aggregation (Brune & Ullrich, 1987; Hobbs & Moncada, 2003).

The enzymatic regulators of these gaseous products, HO and NOS, can be either constitutively expressed or induced. The inducible forms of the enzymes (HO-1 and NOS2/iNOS) both involve gene activation and de novo enzyme protein synthesis (Maines, 1997). Furthermore, NOS2 can be induced by many of the same stimuli that induce HO-1, including cytokines, ROS, and endotoxins (Nathan, 1992; North *et al.*, 1996). Both inducible enzymes are stimulated in highly inflammatory processes (Ignarro, 1993; Willis *et al.*, 1996).

NO, a free radical having a half-life on the order of seconds, is much more labile and unstable than the nonradical CO (Moncada & Higgs, 1995; Cary & Marletta, 2001). Although NO can bind both ferric and ferrous heme, CO can only bind ferrous heme (Pryor *et al.*, 2006). Whereas both NO and CO activate sGC, NO is five times more capable of activating sGC (Stone & Marletta, 1994; Kharitonov *et al.*, 1995). However, some investigators have observed relative relaxation potencies of CO to NO to vary from 1:1000 for rabbit aorta to 1:1 in canine circumflex coronary arteries (Furchgott & Jothianandan, 1991). Whereas constitutive NOS is relatively absent from VSM, constitutive HO is abundant in these cells (Johnson *et al.*, 1999).

Interestingly, the NO and CO pathways exhibit complex patterns of interaction. For example, NO has been found to either inhibit HO activity (Willis *et al.*, 1995; Ding *et al.*, 1999) or enhance it (Motterlini *et al.*, 1996; Leffler *et al.*, 2005). Exogenously administered or endogenously produced NO can selectively induce HO-1 gene expression and CO release in VSM cells (Durante *et al.*, 1997). NO can also cause the release of

free heme from heme proteins (Lipton *et al.*, 1993), which may well lead to increased CO production through increased substrate availability. Maines (Maines, 1997) proposes that the ability of NO to affect HO activity may be accounted for by the free radical nature of NO, such that it may bind to heme, thereby inhibiting or inducing HO activity.

CO can hamper nitric oxide synthesis via eNOS, nNOS and iNOS inhibition (McMillan *et al.*, 1992; White & Marletta, 1992; Matsuoka *et al.*, 1994; Fan *et al.*, 1997; Thorup *et al.*, 1999; Ishikawa *et al.*, 2005) as well as increase NO production via NOS activation (Lim *et al.*, 2005). This duality is also evident in the observation that CO can influence NO release in a biphasic manner; at high CO levels (10μm), NO release from isolated perfused rat afferent arterioles is suppressed, whereas with lower levels (0.01-0.1 μm), NO release is maximal (Thorup *et al.*, 1999). At concentrations of 1-10μm, CO can induce oxidative stress, resulting in the production of peroxynitrite, which further limits the availability of NO (Thom *et al.*, 1997). Furthermore, repeated exposure to brief pulses of CO results in a decreased NO release (Thorup *et al.*, 1999), which may occur by depletion of intracellular pools of heme-bound NO.

5. Postnatal Growth and Maturation: Effects on the Vasculature

A. Introduction

The vascular dysfunction associated with some cardiovascular diseases can begin to develop well before adulthood (Bohlen & Lobach, 1978; Zweifach *et al.*, 1981; Prewitt *et al.*, 1982; Bohlen, 1987; Kunert *et al.*, 2001), so that changes linked to the pathological process become superimposed on the changes in vascular function that normally occur

with juvenile growth. Therefore, to more clearly elucidate the mechanisms of progressive vascular dysfunction under such circumstances, it is necessary to gain a deeper understanding of those changes in vascular function that accompany normal growth.

Early studies in this area were focused almost exclusively on conduit arteries, which differ from resistance vessels in size, function, and local environment. Although these earlier studies have provided valuable information with regards to the elastic muscular transport arteries which serve as a pressure reservoir, these data cannot be generalized to the microcirculation. More information is gradually becoming available on such growth-related changes at the microvascular level.

The rapid postnatal enlargement of organs is accompanied by extensive growth of their arteriolar, capillary and venular networks (Sarelius *et al.*, 1981; Unthank & Bohlen, 1988; Wang & Prewitt, 1991; Linderman & Boegehold, 1996) and increases in microvascular wall mass (Bohlen & Lobach, 1978; Wang & Prewitt, 1991). These structural changes are accompanied by a progressive increase in microvascular pressure and volume flow (Zweifach *et al.*, 1981; Wang & Prewitt, 1991; Linderman & Boegehold, 1998, 1999). Not surprisingly, studies across different species also suggest that the mechanisms responsible for controlling arteriolar resistance and blood flow are not fixed at birth, but instead undergo marked changes during subsequent microvascular growth. The intent of this section is to summarize the current state of knowledge on growth-related changes in the function of both conduit arteries and resistance vessels, and the impact of these latter changes on the regulation of blood flow.

B. Conduit Arteries

i. Endothelium-Dependent Control

Studies on conduit arteries have demonstrated changes in endothelium-dependent control with postnatal growth, although there are inconsistent findings. For example, endothelium-dependent dilator responses to histamine are similar in large mesenteric arteries from 2- and 8-week-old rats, but then decline to smaller responses in arteries from 13-week-old rats (Moritoki *et al.*, 1986). In contrast, the endothelium-dependent relaxation of porcine pulmonary arteries to ACh, BK and the calcium ionophore A23187 progressively increases from 3 to 30 days of age (Zellers & Vanhoutte, 1991).

Activation of endothelial alpha-2 (α_2) receptors does not produce NO in porcine pulmonary arteries at birth or 3 days of age, but does at 10 days of age, and α_2-stimulated NO release is fully developed by 10 weeks (Wilson *et al.*, 1993). ACh elicits larger increases in cGMP formation and greater endothelium-dependent relaxations in pulmonary artery rings from adult sheep than in those from newborn sheep (Kolber *et al.*, 2000), and at least part of this difference may be due to increased expression of sGC in these vessels in the first 2 weeks after birth (Moreno et al, 2005). In contrast, pulmonary artery rings from 2-day-old sheep are more responsive to ACh and BK than those from 1-month old sheep, and an L-arginine analog can reduce these relaxations in 2-day-old vessels but not in 1-month-old vessels, suggesting that these agonists stimulate greater NO release in the younger vessels (O'Donnell *et al.*, 1996).

In the rat, endothelial P_2-purinergic receptors coupled to the L-arginine/NO pathway are nonfunctional in the aorta at 4-6 weeks of age, but become fully functional by 13 weeks of age (Koga *et al.*, 1992). Over this same time period, there is also a

progressive decrease in aortic relaxation to ACh due to the offsetting actions of an endothelium-derived vasoconstrictor prostanoid (Koga *et al.*, 1989). L-NMMA has no effect on the dilation of porcine femoral arteries to ACh at birth, but it significantly reduces these responses at 7-10 days of age (Stoen *et al.*, 1997). Although LNA reduces ACh-induced relaxation of renal arteries in both newborn and adult guinea pigs, this effect is greater in adults (Thompson & Weiner, 1996).

Fluid shear stress induces a graded release of NO that is significantly greater in carotid arteries from adult sheep than those from term fetal lambs, due to the significantly greater ability of fluid shear stress to activate eNOS in adult than in fetal arteries (White *et al.*, 2005). This group also reported that stimulation of NO release by the receptor-independent endothelial activator A23187 was significantly greater in adult than in fetal arteries. When NO release (induced from either fluid shear stress or A23187) was normalized to eNOS abundance between groups, eNOS-specific activity was also significantly greater in adult than in fetal arteries. In contrast, however, stimulation with the receptor-dependent endothelial activator ADP resulted in no difference between fetal and adult arteries in both overall NO release and eNOS-specific activity (White *et al.*, 2005). The authors propose that "fractional" activation of eNOS in response to ADP may be upregulated in fetal compared with adult arteries, which could compensate for the attenuated eNOS activity, thereby maintaining endothelium-dependent responses in arteries from fetal lambs.

Some investigators have looked at NOS protein and expression in various beds with growth. Although age-related differences in NO release in lamb/sheep carotid arteries have been found, no age-related differences in eNOS protein or mRNA levels

were observed (White *et al.*, 2005). However, other investigators have detected a greater nNOS mRNA in immature compared with mature kidneys in pigs (Solhaug *et al.*, 2000).

ii. Smooth Muscle Responsiveness to NO

Smooth muscle responsiveness to NO can also change with postnatal growth. Although femoral arterial rings from newborn and 7-10 day old piglets can relax completely to the NO donor sodium nitroprusside (SNP), the 7-day-old group is more sensitive to SNP than the newborn (Stoen *et al.*, 1997). Similarly, pulmonary artery rings from 10 day old piglets are significantly more responsive to exogenous NO than those from newborn or 3-day-old animals (Wilson *et al.*, 1993). Sensitivity to SNP also increases with age in guinea pig renal arteries (Thompson & Weiner, 1996). In contrast, relaxation to SNP is significantly less in pulmonary artery rings from 1 month old lambs than those from 2 day old lambs (O'Donnell *et al.*, 1996). Similarly, maximum efficacy for relaxation to other NO donors (S-nitroso-N-acetyl-penicillamine and nitroglycerin) decreases with maturation (from 3-7 day old to adult) in sheep basilar and carotid arteries (Pearce *et al.*, 1994). Still others have found no change with growth, as demonstrated by no difference in sensitivity to SNP among porcine pulmonary artery rings from 3-, 10-, and 30-day-old animals (Zellers & Vanhoutte, 1991).

iii. Ion Channel Activity

The activity of various types of plasma membrane K^+ channels, and the effect of this activity on membrane potential and vascular tone, may also change during juvenile growth. Many studies have found that K^+ channel density, mRNA expression, and

sensitivity increase with age. Whole-cell patch-clamp recordings in type I carotid body cells indicate that K^+ current density increases with postnatal age in the rat, such that between 4 and 10 days, there is an enhanced activation of K_{Ca} channels and an increased hypoxic sensitivity to K^+ currents (Hatton *et al.*, 1997). In ovine middle cerebral arteries, dilator responses to the K_{ATP} channel agonist lemakalim are enhanced by maturation, with the -log ED_{50} values for dilation 29 to 43 times greater in adult than in newborn arteries (Pearce & Elliott, 1994). Voltage-gated K^+ channel protein and mRNA expression in ovine pulmonary arteries also increases with age (Cornfield *et al.*, 2000), as does K_{ATP} channel density in rat neuronal tissue (Miller *et al.*, 1991).

Some investigators have found a change in the relative importance of different K^+ channels with maturation. The predominant K^+ channel that regulates resting membrane potential in the ovine pulmonary circulation changes after birth from a K_{Ca} to a K_V channel (Reeve *et al.*, 1998). Maturation also modulates the contribution of K_V, K_{Ca}, and K_{ATP} channels to basal and/ or serotonin (5HT)-induced cerebrovascular tone in ovine middle cerebral arteries, with K_V and K_{Ca} channel currents also more responsive to stretch in adult than in fetal arteries (Teng *et al.*, 2002).

C. Structural Changes during Microvascular Growth

An understanding of microvascular network structure is important for assessing the ultimate functional impact of any growth-related changes in microvascular tone or its regulation. Anatomical modifications of the vasculature can be dramatic during postnatal growth. For example, the hypertrophic growth of skeletal muscle fibers during juvenile

maturation is accompanied by growth of the vasculature (Enesco & Puddy, 1964; Sillau & Banchero, 1977; Ripoll *et al.*, 1979; Aquin *et al.*, 1980). However, Wang and Prewitt (1991) reported that large and small arteriolar density actually decreases from 35 days of age to 132 days of age in the cremaster muscle of Wistar rats, suggesting that the rate of tissue growth is greater than the rate of network growth. These investigators determined that this arteriolar development primarily consists of increases in length, diameter and wall mass of pre-existing vessels, rather than new vessel growth. Similar findings were observed in the rat spinotrapezius muscle, where network growth during maturation (from 3-4 weeks, to 7-8 weeks, to 11-12 weeks) occurs by elongation of pre-existing vessels with angiogenesis only occurring in the distal segments of transverse arteriolar trees (Linderman & Boegehold, 1996). In this latter study, morphometric analysis revealed that there was no significant change in the total number of arcade arteriole segments per network even though the muscle dramatically grew during this time.

Not only are there pronounced reductions in arteriolar density during maturation, but capillary density has also been found to progressively decline. This has been observed in hamster cremaster muscle (Sarelius *et al.*, 1981), and rat soleus (Sillau & Banchero, 1977), tibialis anterior, and gastrocnemius muscles (Sillau & Banchero, 1977; Ripoll *et al.*, 1979). In growing guinea pig soleus and gastrocnemius muscles, capillary density decreases with increased fiber cross-sectional area, but the absolute number of capillaries around the fiber increases, as does myoglobin concentration (Aquin *et al.*, 1980). These increases may at least partially compensate for the lower capillary density to assure adequate O_2 delivery in tissue beds of older animals (Aquin *et al.*, 1980).

Muscle fiber type may affect capillary density, and therefore alterations in fiber type might be an important influence to consider when analyzing the vascular changes that occur during postnatal growth. Changes in fiber type have, in fact, been demonstrated with maturation; in rat tibialis anterior muscle, red fibers increase, but in rat soleus muscle, the percentage of red fibers decreases. These increases or decreases in red fibers affects the capillary to fiber (C: F) ratio, with more red fibers leading to higher C: F ratio and fewer red fibers leading to decreased C: F ratios (Sillau & Banchero, 1977).

D. Hemodynamic Changes during Microvascular Growth

During arteriolar network growth in skeletal muscle, there is a progressive increase in microvascular pressure (Zweifach *et al.*, 1981; Wang & Prewitt, 1991; Linderman & Boegehold, 1998, 1999). In our laboratory, we utilized in vivo microscopy to study proximal arterioles in the spinotrapezius muscle of rats 4-5 wks, 7-8 wks, and 11-12 wks of age. As animals matured, resting diameter and volume flows increased, but resting wall shear rate decreased due to a proportionately greater increase in arteriolar diameters than in blood flow velocity (Linderman & Boegehold, 1998, 1999).

E. Regulation of Microvascular Tone and Tissue Blood Flow during Growth

i. Influences on Arteriolar Tone

In growing skeletal muscle, resting arteriolar tone has been found to either progressively increase (Proctor *et al.*, 1981; Linderman & Boegehold, 1998), or remain relatively constant (Wang & Prewitt, 1991; Linderman & Boegehold, 1999). Even in cases where there is no change in overall resting tone with growth, there is some evidence to indicate that the relative importance of the different mechanisms contributing to that tone (e.g., influences from endothelium-derived vasoactive factors and smooth muscle myogenic activity) may vary greatly over this period. Our laboratory previously found that L-NMMA treatment reduces arteriolar diameters in the spinotrapezius muscle of more juvenile rats, but not younger rats (Linderman & Boegehold, 1998; Linderman & Boegehold, 1999; Nurkiewicz & Boegehold, 2004), whereas meclofenamate reduces resting arteriolar diameters in rats of all ages (Linderman & Boegehold, 1998). These findings suggest that continuously-released NO and vasodilator prostanoids exert a moderating influence on arteriolar tone in the juvenile spinotrapezius muscle, whereas prostanoids, but not NO, exert such an influence in the younger animals. Similarly, NOS inhibition with L-NAME has no effect on the diameter of pial arterioles in newborn pigs (Zuckerman *et al.*, 1996; Willis & Leffler, 2001), but it reduces the diameter of these arterioles in juvenile pigs (Willis & Leffler, 2001). L-NAME significantly constricts cerebral resistance arteries from adult mice, but not those from neonatal mice (Geary *et al.*, 2003). In contrast, inhibition of NOS increases renal vascular resistance in conscious lambs, but this effect is greatest at 1 week of age and then progressively declines at 3 and 6 weeks of age (Sener & Smith, 2001). These heterogeneous observations suggest that synthesis of and responses to various endothelium-derived mediators can change with

age, and that these changes in vascular tone regulation may not be consistent among different vascular beds or species.

As in larger vessels, postnatal growth of the microvasculature can be accompanied by significant changes in the endothelium-dependent mechanisms that mediate acute changes in vascular tone. For example, the effectiveness of the endothelium-dependent dilators ACh or histamine in reducing mesenteric vascular resistance in the rat increases from ages 3 weeks to 3 months (Fleisch & Spaethe, 1981). Similarly, DeMey and Gray reported a progressive increase in the responsiveness of isolated rat mesenteric resistance arteries to ACh or histamine during the first two weeks of life. Furthermore, these investigators found that BK constricted resistance arteries from rats less than 4 weeks of age, but elicited an endothelium-dependent dilation of those from older rats. In the microcirculation of the rat sciatic nerve, ACh induces a non-NO, non-prostanoid-dependent vasodilatation which is larger in 1-2 week old rats than in 18-20 week old rats (Thomsen *et al.*, 2002).

In pial arterioles of juvenile pigs, ACh induces an early prostanoid-associated constriction followed by a NO-associated dilation, whereas in pial arterioles of newborn pigs, ACh induces only a brief prostanoid-associated vasoconstriction (Zuckerman *et al.*, 1996). Although the dilation of these vessels to BK relies on the COX and NOS systems in both juvenile and newborn pigs, the juvenile vessels are more dependent on NO, whereas the newborn vessels are more dependent on prostanoids (Willis & Leffler, 2001). Such a finding could reflect age-related differences in COX or NOS expression, or in the activity of these enzymes. Using cultured microvascular ECs from newborn and adult pig cortex, Parfenova *et al.* (2000) found no differences in COX expression or activity

between these groups, but did find higher NOS expression and activity in adult cells than in newborn cells. Cerebrovascular dilations to hypercapnia and histamine are also prostanoid dependent in the newborn pig, with NO assuming an increasing role in these responses during subsequent growth (Willis & Leffler, 1999).

Linderman and Boegehold (1999) found no age-related differences in overall arteriolar responsiveness to locally applied ACh in the spinotrapezius muscle of rats 4-5 wks, 7-8 wks, and 11-12 wks of age. However, they did report that the L-NMMA-sensitive portion of the ACh response was smaller in mature rats than in weanling rats. Although elevation of flow-related shear stress resulted in arteriolar dilation in juvenile rats, it had no effect in the weanling rats. In a subsequent study (Nurkiewicz & Boegehold, 2004), arterioles from weanling and juvenile rats exhibited identical dilator responses to the Ca^{2+} ionophore A23187, and these responses were equally sensitive to L-NMMA in both age groups. However, the Ca^{2+}-independent agonists VEGF and simvastatin elicited dose-dependent, L-NMMA sensitive, arteriolar dilations in juveniles, but no or only minimal responses in the weanling group. These findings suggest that Ca^{2+}-dependent signaling pathways for NO release do not change with rapid arteriolar network growth during juvenile maturation, whereas Ca^{2+}-independent NO release is absent in the weanling endothelium but these pathways become functional during maturation.

Numerous studies indicate that the intrinsic responsiveness of arteriolar smooth muscle to NO does not change during microvascular growth, either in skeletal muscle (Li & Joshua, 1993; Linderman & Boegehold, 1999) or other vascular beds (Fleisch & Spaethe, 1981; Willis & Leffler, 1999). This suggests that age-related changes in the

contribution of NO to the regulation of arteriolar diameter are primarily attributable to functional changes in endothelial cells rather than VSM cells.

The myogenic response contributes largely to the establishment of basal vascular tone, and to autoregulation of blood flow and capillary hydrostatic pressure (Davis & Hill, 1999; see Section 2B above). Although resting tone is due in part to myogenic activity, diameter changes in response to an abrupt increase or decrease in transmural pressure more clearly define myogenic responsiveness (Su *et al.*, 2003). As some studies suggest that endothelium-dependent control may not be fully developed in early stages of postnatal growth, perhaps myogenic activity might be enhanced during this time period. Some authors have found that there is an increased intensity of the myogenic response in 1-day old piglet mesenteric resistance arteries when compared to 10-day old vessels (Su *et al.*, 2003). Pressure-induced myogenic tone is regulated similarly in neonatal and adult mouse cerebral arteries, but recent work by Geary and colleagues (2003) indicates that the modulation of this activity by endothelial vasoactive factors is age dependent. In that study, whereas L-NAME treatment significantly reduced arterial diameters at all pressures (ranging from 20 to 80 mmHg) in adults, it had no effect on neonatal arteries at any pressure. Furthermore, although indomethacin treatment had no effect on either adult or neonatal arteries alone, subsequent addition of L-NAME significantly enhanced arterial tone over the entire pressure range in both groups. In arteries pre-treated with L-NAME, subsequent addition of indomethacin enhanced tone to an even greater extent at all pressures in adult arteries, but had no further effect in the neonatal arteries. Finally, during combined NOS/COX inhibition, intrinsic tone was significantly greater in

neonatal than adult arteries, but when the endothelium was removed, tone was similar in neonatal and adult arteries at all pressures (Geary *et al.*, 2003).

ii. Tissue Blood Flow and Oxygen Delivery

Various factors that influence tissue O_2 delivery and consumption have been found to change with growth and development. For example, capillary hematocrit (Sarelius *et al.*, 1981) and skeletal muscle oxidative metabolism (Hudlicka, 1985) decrease with maturation. Capillary red cell content (number per unit length) and maximal red cell flux also decrease with age (Berg & Sarelius, 1996). It appears that young animals have superior arteriolar sensitivity to O_2 and are able to maintain tissue PO_2 during muscle contraction more precisely than older animals (Proctor *et al.*, 1981). Whereas resting tissue PO_2 over a wide range of superfusate PO_2 levels is not different in the cremaster muscle from hamsters aged 32, 60 and 80 days, increased metabolic demand, via muscle stimulation, causes tissue PO_2 to decrease significantly in adult animals, but not in the immature or intermediate groups. For any given increase in tissue PO_2, the arteriolar constriction is also age dependent, with the oldest animals demonstrating the smallest response (Proctor *et al.*, 1981).

Metabolic requirements are constantly changing throughout postnatal growth, and skeletal muscle blood flow is distributed according to metabolic demand (Hudlicka, 1969). We and others have found an increase in total network blood flow in rat spinotrapezius muscle (Linderman & Boegehold, 1999) and rat cremaster muscle (Wang & Prewitt, 1991). However, as the muscle mass increases dramatically during this period of growth, blood flow per gram of tissue actually decreases, which is consistent with

numerous studies documenting a progressive decrease in total skeletal muscle blood flow during maturation (Hudlicka, 1985). With postnatal growth, animals also experience increases in cardiac output (Teitel & Rudolph, 1985), myoglobin content (Aquin *et al.*, 1980), and body temperature (Mueggler *et al.*, 1979), and overall decreases in arteriolar wall shear stress (Linderman & Boegehold, 1999). Furtherore, fetal hemoglobin is gradually replaced by adult hemoglobin (Teitel & Rudolph, 1985), such that decreases in total hemoglobin O_2 affinity also occur (Lister *et al.*, 1979).

Whereas some suggest that younger animals may have a superior blood flow regulatory capability (Proctor *et al.*, 1981), Berg and Sarelius (1996) have argued that despite all of the changes in RBC flow variables which occur with maturation, the capacity for networks to deliver O_2 remains constant; even with variability in network flow parameters during maturation, mean capillary wall PO_2 remains within a narrow range both at rest and with maximal dilation. These investigators attribute this to the more extensive recruitment of capillary networks (Berg & Sarelius, 1995), and the higher capacity of recruited networks to deliver O_2 in adults compared to younger animals.

It is important to keep in mind that the vascular network is not static. During development the vasculature can accommodate increased metabolic demands or decreased O_2 supply via a number of methods. For example, changes in the activity of oxidative enzymes, or volume density of mitochondria may allow the network to adapt (Hoppeler *et al.*, 1981; Hudlicka, 1985). Perhaps local hypoxia might trigger an induction of capillary growth (Adair *et al.*, 1990), thereby allowing adaptation to metabolic needs. Others have suggested flow-induced shear stress or local changes in

growth factors as being possible stimuli for network development (Wang & Prewitt, 1991; Dawson & Hudlicka, 1993).

6. Conclusion

This literature review highlights the challenges associated with interpreting findings from different vascular beds and species, and synthesizing such heterogeneous findings into a cogent understanding of how vascular function may change with postnatal growth. Much of the literature is focused on the pulmonary vasculature, which is substantially different from that of other vascular beds. The fetal pulmonary vasculature is a high-tone, low flow system, which changes quickly at birth to accommodate an 8-10-fold increase in blood flow and an increase in intravascular pressure (Dawes *et al.*, 1953; Cassin *et al.*, 1964). Given all these differences among species, vessel sizes, and preparations, caution must be taken when synthesizing a coherent analysis of postnatal vascular changes. However, Table 2 summarizes some of the general trends that have been observed with growth and development in the larger and smaller vessels.

Investigators have studied mechanisms regulating arteriolar tone across a wide variety of tissues and organs, and much has been learned about these complex mechanisms. However, there is much less known about how these mechanisms may change during postnatal growth and maturation. A thorough understanding of the normal changes in endothelial/ smooth muscle signaling pathways and vascular mechanics during postnatal growth may lead to a better understanding of the pathophysiological changes that can also occur over this time in some disease states.

Table 2: Changes in endothelium-dependent vascular control during postnatal growth: A summary of findings from the literature

Animal	Tissue	Stimulus	Change in Vascular Response (↑, ↓, ↔)	Ages Studied	Investigators
Rat (Sprague-Dawley)	Mesenteric artery	ACh, histamine	↑ with age	3 wks, 3 mo, 6 mo	Fleisch & Spaethe, 1981
Rat (Wistar-Kyoto)	Mesenteric artery	ACh, histamine	↑ during 1st 2 wks, but are then maintained throughout adult life	4, 7, 14, 28, 80, 280 days	DeMey & Gray, 1985
Rat (Wistar-Kyoto)	Mesenteric artery	BK	Constriction until 4 wks, then relaxation	4, 7, 14, 28, 80, 280 days	DeMey & Gray, 1985
Rat (Wistar)	Mesenteric artery	histamine	↓ with age	2-8 weeks, 13 wks, 56 wks	Moritoki *et al.*, 1986
Pig	Pulmonary artery	ACh, BK, A23187	↑ with age	3, 10, 30 days	Zellers & Vanhoutte, 1991
Sheep	Pulmonary artery	ACh	↑ with age	fetal, newborn, adult	Kolber *et al.*, 2000
Sheep	Pulmonary artery	A23187	↑ with age	Fetal, 4 wks	Mata-Greenwood *et al.*, 2006
Sheep	Pulmonary artery	ACh, BK	↓ with age	2 d, 1 mo	O'Donnell *et al.*, 1996
Rat (Wistar-Kyoto)	Aorta	ACh	↓ with age	4-6 wks, 3-6 mo, 12-25 mo	Koga *et al.*, 1989

(continued on next page)

Animal	Tissue	Stimulus	Change in Vascular Response (↑, ↓, ↔)	Ages Studied	Investigators
Rat (Wistar-Kyoto)	Aorta	ATP	Endothelium-independent relaxation (4-6 wks) to endothelium-dependent relaxation (13-14 wks)	4-6, 9-10, 13-14 wks	Koga *et al.*, 1992
Dog	Aorta	ACh	↓ with age	fetal, newborn, adult	Torok & Gerova, 1996
Pig	Pial arteriole	ACh, BK	Newborn relies on prostaglandins, juvenile relies more on NO	Newborn, 3-4 mo	Zuckerman *et al.*, 1996; Willis & Leffler, 2001
Rat (Sprague-Dawley)	Spinotrapezius muscle	ACh:	No difference in response, although L-NMMA-sensitive part of this response was smaller in mature rats	4-5, 7-8, 11-12 wks	Linderman & Boegehold, 1999
		Increased shear stress:	No response in weanlings, but dilation in juveniles		
Rat (Sprague-Dawley)	Spinotrapezius muscle	A23187:	No difference in responses	4-5 wks, 7-8 wks	Nurkiewicz & Boegehold, 2004
		VEGF/Simvastatin:	No response in weanlings, but dose-dependent responses in juveniles		

7. Dissertation Objectives

Because there are few studies that have investigated how postnatal growth affects microvascular function, we undertook these experiments, in part, to help fill a critical gap in the literature. Specifically, we wanted to gain further insight into growth-related changes in the control of skeletal muscle arterioles.

A clearer understanding of how the endothelium and smooth muscle work together to translate biochemical signals into vascular responses during blood vessel growth may ultimately lead to the identification of new therapeutic targets for the endothelial dysfunction associated with cardiovascular diseases that can begin to develop well before adulthood.

The specific aims of each study within this project are listed below.

Study 1

We undertook this study to more completely understand how growth affects endothelium-dependent control of skeletal muscle arterioles. This was the first step toward a greater appreciation of how microvascular function is altered during postnatal development, and was the springboard for all subsequent studies.

Study 2

Because H_2O_2 can serve as an important endothelium-derived vasoactive factor in the cerebral microcirculation of newborn pigs, we undertook this study to determine if H_2O_2 also mediates endothelium-dependent dilation in skeletal muscle arterioles of young

rats. A second aim of this study was to determine if there are any age-related differences in the mechanism by which H_2O_2 can influence arteriolar smooth muscle tone.

Study 3

Because CO has been identified as an important vasoactive factor in the cerebral microcirculation of newborn pigs, we undertook this study to determine if CO could be playing a similar role in the endothelium-dependent dilation of skeletal muscle arterioles from young rats. A second aim of this study was to determine if there the mechanism by which CO influences arteriolar tone undergoes a change during rapid juvenile growth.

Study 4

We previously found that gracilis muscle arterioles from weanling rats develop a significantly higher level of resting tone than arterioles from juvenile rats, suggesting that arteriolar smooth muscle responsiveness to myogenic stimuli may be greater in the younger rats. We therefore undertook this study to more rigorously investigate potential alterations in myogenic activity that may occur during microvascular network maturation in skeletal muscle arterioles.

II. STUDY 1: Growth-Dependent Changes in Endothelial Factors Regulating Arteriolar Tone

Abstract

Previous studies from this laboratory suggest that during maturation, rapid microvascular growth is accompanied by changes in the mechanisms responsible for regulation of tissue blood flow. To further define these changes, we studied isolated gracilis muscle arterioles from weanling (~25 days) and juvenile (~44 days) Sprague-Dawley rats to test the hypothesis that endothelial mechanisms for the control of arteriolar tone are altered with growth. Responses to the endothelium-dependent dilator acetylcholine (ACh) were greater in weanling arterioles (WA) than in juvenile arterioles (JA), whereas there were no consistent differences between age groups in arteriolar responses to other endothelium-dependent agonists (A23187, VEGF and simvastatin). Inhibition of nitric oxide synthase (NOS) with L-NAME attenuated ACh-induced dilation in JA, but not in WA. In JA, combined inhibition of NOS and cyclooxygenase (with indomethacin) reduced the dilator responses to ACh and simvastatin by ~90% and ~70%, respectively, but had no effect in WA. Cytochrome P450 epoxygenase inhibition (with PPOH) had no effect on responses to ACh or simvastatin in either age group. Inhibition of Ca^{2+}-activated or ATP-dependent potassium channels (with TEA or glibenclamide, respectively) reduced these arteriolar responses in JA but not WA. These findings suggest that in fully grown microvascular networks, endothelium-dependent arteriolar dilation is mediated by the combined release of endothelial nitric oxide and vasodilator

prostanoids, and in part through activation of K_{Ca} and K_{ATP} channels. However, during earlier microvascular growth, this dilation is mediated by other factors yet to be identified. This may have significant implications for the regulation of tissue perfusion during microvascular development.

Introduction

There is mounting evidence to suggest that postnatal growth of the microvasculature is accompanied by progressive changes in a number of factors that can influence arteriolar tone and blood flow (Zellers & Vanhoutte, 1991; Olesen *et al.*, 1994; Linderman & Boegehold, 1996, 1999; Nurkiewicz & Boegehold, 2004). During this rapid growth phase, the overall impact of any phenotypic changes in the endothelium or vascular smooth muscle could also be amplified or otherwise modulated by concomitant changes in arteriolar wall structure and/or local hemodynamic forces (Zweifach *et al.*, 1981; Wang & Prewitt, 1991; Linderman & Boegehold, 1999). Profound growth-related changes also occur at the capillary level. For example, in growing skeletal muscle, there is an increase in total tissue blood flow and in the absolute number of capillaries (Aquin *et al.*, 1980; Wang & Prewitt, 1991), but a decrease in capillary density, capillary hematocrit, and individual capillary blood flow (Sarelius *et al.*, 1981). Some of these changes may be necessary for the microvasculature to continually adapt to the changing metabolic demands associated with rapid tissue growth (Berg & Sarelius, 1996).

Growth-related changes in endothelial function were first documented in conduit arteries. Koga and colleagues reported that in rat aorta, endothelial P_2 purinergic receptors coupled to the L-arginine/nitric oxide (NO) pathway are nonfunctional at 4-6 weeks of age, but become fully functional by 13 weeks (Koga *et al.*, 1992). This same

group also found that rat aorta undergoes a progressive decline in its overall responsiveness to acetylcholine (ACh) from approximately 4 weeks to 4 months of age (Koga *et al.*, 1989). Not surprisingly, such changes in responsiveness can begin soon after birth. For example, the endothelium-dependent relaxation of small porcine pulmonary arteries to ACh and BK increases from 3 to 10 days of age (Zellers & Vanhoutte, 1991). In some cases, these changes may also be biphasic; BK causes endothelium-dependent contraction of small rat mesenteric arteries until 4 weeks of age, but endothelium-dependent relaxation of these vessels in older animals (DeMey & Gray, 1985).

Less is known about growth-related changes in endothelial function at the microvascular level. Willis and Leffler (2001) have reported that although pial arterioles in newborn and juvenile pigs show similar responsiveness to BK, the predominant mediators of this dilation are endothelium-derived prostanoids in newborns and endothelium-derived NO in juveniles. Previous studies in our laboratory demonstrated that although arteriolar responsiveness to ACh in rat spinotrapezius muscle is similar at 4 and 8 weeks of age, these responses are more sensitive to L-NMMA in the 4 week-old animals, suggesting a greater contribution of NO to the overall dilation (Linderman & Boegehold, 1999). We have also reported that vascular endothelial growth factor (VEGF) and simvastatin elicit a dose-dependent dilation of these arterioles at 8 weeks of age, but not at 4 weeks of age (Nurkiewicz & Boegehold, 2004). These two agonists stimulate endothelial NO release via activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, leading to direct phosphorylation and increased activity of eNOS at low cytoplasmic Ca^{2+} levels (Kureishi *et al.*, 2000). Therefore, Ca^{2+}-independent

signaling pathways for endothelial NO release may not yet be functional in arterioles of the younger animals.

We undertook the current study to gain further insight into these growth-related changes in the endothelium-dependent control of skeletal muscle arterioles. In this study, arterioles were isolated and studied *in vitro* to eliminate the possibility of any indirect and potentially complicating effects on arteriolar behavior due to circulating or tissue-derived factors. A clearer understanding of how the endothelium translates biochemical signals into vascular responses during blood vessel growth may ultimately lead to the identification of new therapeutic targets for the endothelial dysfunction associated with cardiovascular diseases that can begin to develop well before adulthood.

Materials and Methods

Animals: All surgical and experimental procedures were approved by the West Virginia University Animal Care and Use Committee. Experiments were performed on isolated gracilis muscle arterioles from male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) of two age groups: 3-4 weeks ("weanlings") and 6-7 weeks ("juveniles").

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight), with supplemental anesthetic (10% of original dose) administered if needed. The right carotid artery was cannulated with polyethylene tubing (PE-10 for weanlings, PE-50 for juveniles) for measurement of mean arterial pressure, which was assessed before removal of gracilis arteriole.

Preparation of Isolated Vessels: An arteriolar branch of the femoral artery supplying the gracilis muscle was removed, handling only the surrounding connective tissue to minimize vessel stretching or damage. The rat was then sacrificed immediately following vessel removal by intracardiac injection of sodium pentobarbital. The vessel was then placed in warmed physiological salt solution (PSS) equilibrated with 21% O_2, 5% CO_2, and 74% N_2 and having the following composition (mM): 119 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 1.6 $CaCl_2$, 1.18 NaH_2PO_4, 24 $NaHCO_3$, 0.026 EDTA, and 5.5 glucose. After isolation, each vessel was prepared for *in vitro* video microscopy as previously described (Fredricks *et al.*, 1994a). Briefly, the vessel was mounted in a heated (37º C) chamber that allowed its lumen and exterior surface to be perfused and superfused, respectively, with PSS from separate reservoirs. The vessel was cannulated at both ends with glass micropipettes (50- and 70-μm tip diameters for weanling and juvenile vessels, respectively) and secured to the inflow and outflow pipettes using 9-0 nylon suture. Any side branches were ligated with a single strand teased from 6-0 silk suture. The inflow pipette was connected to a reservoir perfusion system for control of intralumenal pressure and flow. The vessel was then extended to its *in situ* length and equilibrated at 80% of the animal's mean arterial pressure to approximate its *in vivo* perfusion pressure (DeLano *et al.*, 1991).

Vessel diameter was measured using an onscreen video micrometer. Any vessel that did not demonstrate endothelial viability, as judged by responsiveness to 10^{-7} M ACh, was not used in the study. Diameter measurements were made under static, zero-flow conditions after a 30-minute equilibration period with continuous perfusion. Resting vascular tone was calculated as $(\Delta D/D_{max})\bullet 100$, where ΔD is the diameter

increase from rest in response to Ca^{2+}-free PSS (30-40 minute equilibration with Ca^{2+}-free bath solution and no lumenal flow), and D_{max} is the maximum diameter measured under these conditions.

Agonists: All agonists were dissolved in PSS unless otherwise noted. ACh (Sigma Chemical, St. Louis, MO), at bath concentrations of 10^{-5}, 10^{-6}, or 10^{-7} M, and the Ca^{2+}-ionophore A23187 (Sigma) at a bath concentration of 10^{-9} M, were used to assess arteriolar capacity for Ca^{2+}-dependent endothelial NO formation (Ungvari *et al.*, 2001). To produce the desired concentration, 1 milligram A23187 was first dissolved in 50 µl of dimethylsulfoxide (DMSO) and then diluted with Dulbecco's phosphate-buffered saline (PBS) before addition to the bath.

VEGF (BD Biosciences, Lexington, KY) and simvastatin (Merck Research Laboratories, Rathway, NJ) were used to assess arteriolar capacity for Ca^{2+}-independent endothelial NO formation (Nurkiewicz & Boegehold, 2004). VEGF was dissolved in PBS at a concentration of 5 µg/ml and added directly to the bath. Simvastatin was activated by alkaline lysis (5.25 ml of 0.1 N NaOH per 140 mg, dissolved in 3.5 ml of ETOH) at 50ºC for 2 h. The resulting solution was then diluted to a volume of 35 ml with PBS, and neutralized to pH 7.4 with HCl. One-ml aliquots of this solution were then serially diluted with PBS, producing a final bath concentration of 10^{-4}, 10^{-5}, 10^{-6}, or 10^{-7} M.

To assess intrinsic vascular smooth muscle responsiveness to NO, the endothelium-independent vasodilator sodium nitroprusside (SNP, Sigma) was used at bath concentrations of 10^{-5} M and 10^{-7} M.

Endothelial Denudation: To determine the role of the endothelium in mediating arteriolar responses to ACh and simvastatin, the endothelium was removed by mechanical abrasion (Uluoglu & Zengil, 2003). The pipette tips on both sides of the vessel were gently advanced through the vessel lumen at least three times to ensure elimination of the endothelium. In order to ensure that the endothelial denudation did not adversely affect underlying arteriolar smooth muscle, vasoconstrictor responses to 10^{-5} M phenylephrine and vasodilator responses to 10^{-5} M SNP were assessed before and after the denudation procedure, and only those vessels with unchanged responses to both agonists were included in this study.

Inhibition of NOS, Cyclooxygenase, and Cytochrome P450 Enzymes: To determine the contribution of endothelial NO production to arteriolar dilation, the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) was added to the bath at a concentration of 10^{-4} M (Frisbee *et al.*, 2001). To assess the contribution of vasodilator prostanoids, the cyclooxygenase inhibitor indomethacin was added to the bath at a concentration of 10^{-6} M (Fredricks *et al.*, 1994a). To assess the specific contribution of cytochrome P450 (CP450) epoxygenase-derived metabolites of arachidonic acid, the selective suicide substrate inhibitor 6-(2-propargyloxyphenyl) hexanoic acid (MS-PPOH) was added to the bath at a concentration of 10^{-5} M (Wang *et al.*, 1998). All inhibitors were purchased from Sigma.

Inhibition of Potassium Channels: Since endothelium-derived hyperpolarizing factors (EDHFs) modulate arteriolar tone through activation of potassium channels (Welsh & Segal, 2000; Coleman *et al.*, 2001), the contribution of EDHFs to arteriolar dilation was assessed by using tetraethylammonium (TEA) (Fredricks *et al.*, 1994b;

Lombard *et al.*, 1999; Fallet *et al.*, 2001), or the antidiabetic sulphonylurea, glibenclamide (Lombard *et al.*, 1999). We used a 10^{-3} M bath concentration of TEA to selectively block Ca^{2+}-activated K^+ (K_{Ca}) channels (Nelson & Quayle, 1995), and 10^{-6} M glibenclamide to selectively block K_{ATP} channels (Standen *et al.*, 1989).

To independently assess the effectiveness of these inhibitors in blocking potassium channels, we used 1-(2'-hydroxy-5'-trifluoromethylphenyl)-5-trifluoromethyl-2(3*H*)benzimidazolone (NS 1619), a synthetic BK_{Ca} channel opener, (Fallet *et al.*, 2001) (Olesen *et al.*, 1994), and pinacidil, a nonspecific K_{ATP} channel opener (24). NS 1619 was dissolved in ETOH to a produce a 69 mM stock solution, which was then diluted to a 30 μM bath concentration with PSS (Olesen *et al.*, 1994; Fallet *et al.*, 2001). A 10^{-2} M stock solution of pinacidil (in 100% DMSO) was diluted with PSS to produce a final bath concentration of 10^{-5} M (Mabanta *et al.*, 2006). At their final bath concentrations, neither the ETOH vehicle nor the DMSO vehicle had any effect on resting arteriolar diameters, indicating that they were not intrinsically vasoactive. After the response to either potassium channel opener was assessed under control conditions, the response was re-assessed after treatment of the vessel with TEA and glibenclamide separately and then in combination.

Data and Statistical Analyses: To account for age-related differences in resting and passive arteriolar diameters (see Table 1), dilator responses were expressed as percent of maximum dilation ([diameter change/(D_{max} – control diameter)] x 100).

All data are presented as mean ± SE. For all analyses, a probability value of $p < 0.05$ was considered to be statistically significant. Dilation in response to Ca^{2+}-free PSS is expressed as percent increase from control diameter. Differences between the means

of individual experimental groups were determined by ANOVA/Newman-Keuls test, or by an unpaired Student's *t*-test when two means were compared.

Results

General characteristics of all rats from which vessels were removed are reported in Table 1. Age, body weight, and mean arterial pressure were significantly greater in juvenile rats than in weanling rats. Table 1 also summarizes the general characteristics of all vessels studied. Resting and passive diameters of arterioles from juvenile rats were significantly greater than those of arterioles from weanling rats, whereas the level of spontaneous tone that developed with vessel pressurization was significantly less in juvenile arterioles than in weanling arterioles.

All agonists dilated both weanling and juvenile arterioles, with a slightly smaller dilation to ACh seen in juvenile arterioles (Figure 1). However, there were no consistent differences between age groups in the magnitude of dilation to the other agonists, except for a moderately smaller response of juvenile arterioles to 10^{-7} M simvastatin (Figure 1). Neither L-NAME nor indomethacin treatment, alone or in combination, changed the resting diameters of either juvenile or weanling arterioles (data not shown). For juvenile arterioles, the mean response to a concentration of each agonist that initially caused close to half-maximal dilation was reduced by 60-90% in the presence of L-NAME (Figure 2, top panel). Indomethacin significantly reduced only those responses to A23187 and simvastatin (by 90% and 63%, respectively). In contrast, the responses of weanling arterioles to each of these agonists were unaffected by L-NAME or indomethacin (bottom panel).

The remainder of this study was focused exclusively on arteriolar responses to ACh and simvastatin. As shown in Figure 3, removal of the endothelium completely abolishes the arteriolar dilation to each agonist in both age groups, verifying that these responses are endothelium-dependent. Simultaneous exposure to L-NAME and indomethacin reduced the mean dilator responses of endothelium-intact juvenile arterioles to ACh and simvastatin by ~90% and ~70%, respectively (Figure 4, top panel), but had no effect on the responses of weanling arterioles to these agonists (bottom panel). In contrast, juvenile and weanling arterioles exhibited similar responses to two different concentrations of SNP (Figure 5).

PPOH had no effect on the resting diameters of either juvenile or weanling arterioles (data not shown), and PPOH by itself had no effect on the responses of juvenile or weanling arterioles to either ACh or simvastatin (Figure 6). Furthermore, whereas juvenile arteriolar responses to ACh and simvastatin were greatly reduced by PPOH + L-NAME + indomethacin, the magnitude of this effect was similar to that of L-NAME + indomethacin without PPOH (Figure 4, top panel). The combination of PPOH, L-NAME and indomethacin had no effect on the responses of weanling arterioles to ACh or simvastatin (Figure 6, bottom panel).

Glibenclamide had no effect on the resting diameters of either weanling or juvenile arterioles, whereas 1 mM TEA reduced the resting diameters of both juvenile and weanling arterioles by approximately the same amount (10-12%) (Table 2). The combination of glibenclamide + TEA significantly reduced the resting diameters of both juvenile and weanling arterioles by approximately the same amount as TEA alone. Glibenclamide alone had no significant effect on the dilation of juvenile arterioles to

ACh, but reduced the dilation of these vessels to simvastatin (Figure 7, left). In contrast, TEA alone markedly reduced the responses of these vessels to both agonists (Figure 7, center). Consistent with glibenclamide's lack of effect on ACh responses, glibenclamide and TEA in combination reduced these responses by the same magnitude as TEA alone (Figure 7, right). Glibenclamide + TEA also abolished juvenile arteriolar responses to simvastatin. Neither glibenclamide nor TEA, alone or in combination, reduced the dilation of weanling arterioles to either agonist (Figure 7, bottom panels). Responses of weanling arterioles to ACh also remained unchanged when bath TEA concentration was increased to 5 mM, although responses to 10^{-5} M simvastatin were reduced (by 44.5 ± 0.4%) under these conditions (data not shown).

Treatment with glibenclamide significantly attenuated responses to the K_{ATP} channel opener pinacidil in both weanling and juvenile arterioles (Table 3). Although treatment with 1mM TEA also attenuated the pinacidil responses of weanling arterioles, it had no effect on these responses in juvenile arterioles. Treatment with both glibenclamide and TEA attenuated pinacidil responses in both groups. Treatment with glibenclamide had no effect on arteriolar responses to the BK_{Ca} channel opener NS 1619 in either group, whereas TEA abolished these responses in both groups (Table 4). As expected, the effect of glibenclamide + TEA on NS 1619-induced dilation (complete abolition) was the same as that of TEA alone in both groups.

Table 1: General characteristics of all rats and vessels used in this study

Animal Characteristics	**Weanlings**	**Juveniles**
N	68	52
Age (days)	25.3 ± 0.3	42.0 ± 0.7 *
Body Weight (g)	63.1 ± 1.3	181.4 ± 2.9 *
MAP (mmHg)	82.3 ± 0.9	100.2 ± 1.4 *
Vessel Characteristics	**Weanlings**	**Juveniles**
n	68	52
Resting Diameter (μm)	44.1 ± 1.5	61.6 ± 2.1 *
Passive Diameter (μm)	67.8 ± 1.9	85.6 ± 2.2 *
Resting Vascular Tone (%)	34.5 ± 1.6	28.0 ± 1.5 *

Values are given as means ± SE. * $P<0.05$ vs. weanling group.

Table 2: Summary of vessel diameters under control conditions and after treatment with glibenclamide and TEA separately and in combination

Arteriolar Diameter (µm)

	Control *[n=8]*	***Glib***	***Control*** *[n=5-6]*	***TEA***	***Control*** *[n=8]*	***Glib + TEA***
Weanlings	*39.8±4.0*	*39.6±3.8*	*42.3±2.5*	*38.3±2.6**	*39.8±4.0*	*34.2±3.6**
Juveniles	*55.3±3.7*	*55.2±3.9*	*56.7±3.7*	*49.8±4.5**	*55.3±3.7*	*47.8±3.0**

Values are given as means ± SE. * $P<0.05$ vs. Control.

Table 3: Responses to the K_{ATP} channel opener pinacidil

	Pinacidil *[n=5-6]*	***Pinacidil + Glib***	***Pinacidil*** *[n=4]*	***Pinacidil + TEA***	***Pinacidil*** *[n=5-6]*	***Pinacidil + Glib/TEA***
Weanlings	*56.7±9.3*	*9.4±3.0**	*50.2±6.6*	*24.7±7.1**	*56.7±9.3*	*13.0±5.0**
Juveniles	*56.7±5.4*	*14.7±4.5**	*43.6±3.5*	*33.7±6.0*	*56.7±5.4*	*11.5±1.1**

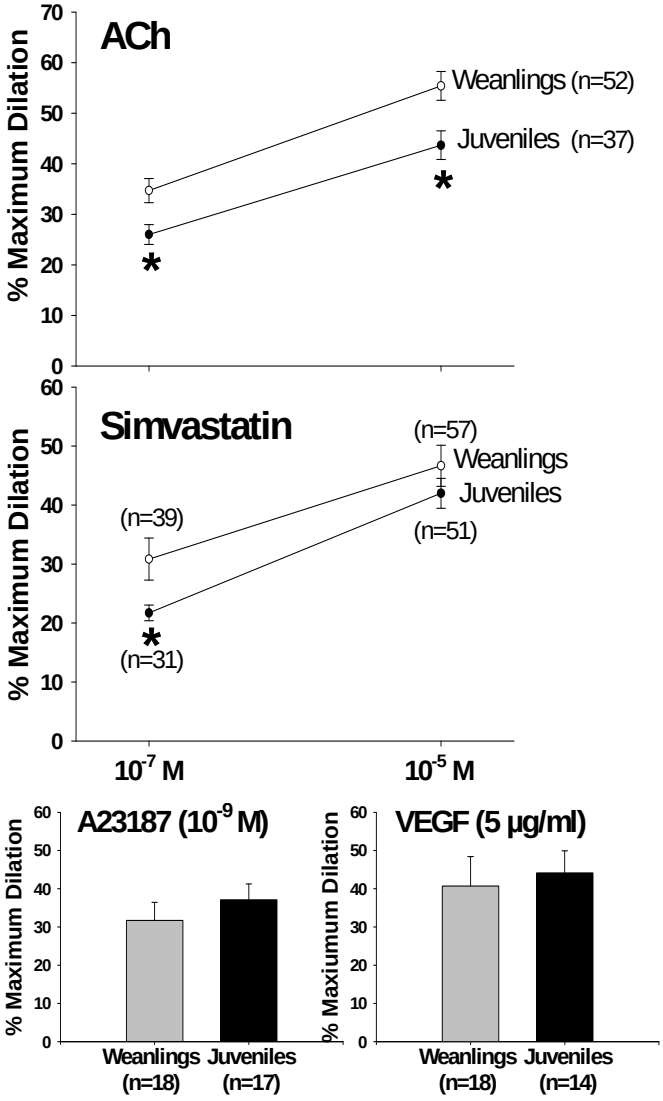
Responses of arterioles (in % maximum dilation) from weanling and juvenile rats to pinacidil (10^{-5} M) before and after inhibition of K_{ATP} channels with 10^{-6} M glibenclamide (Glib) or inhibition of K_{Ca} channels with 1 mM TEA, separately and in combination. Values are given as means ± SE. * $P<0.05$ vs. preceding response to pinacidil.

Table 4: Responses to the BK_{Ca} channel opener NS 1619

	NS 1619 *[n=5]*	***NS 1619 + Glib***	***NS 1619*** *[n=4]*	***NS 1619 + TEA***	***NS 1619*** *[n=5]*	***NS 1619 + Glib/TEA***
Weanlings	*30.6±4.4*	*32.6±6.9*	*58.0±22.1*	*3.7±6.4**	*30.6±4.4*	*-1.5±2.6**
Juveniles	*31.3±2.6*	*31.9±2.8*	*32.5±11.5*	*-15.0±4.7**	*31.3±2.6*	*1.9±1.9**

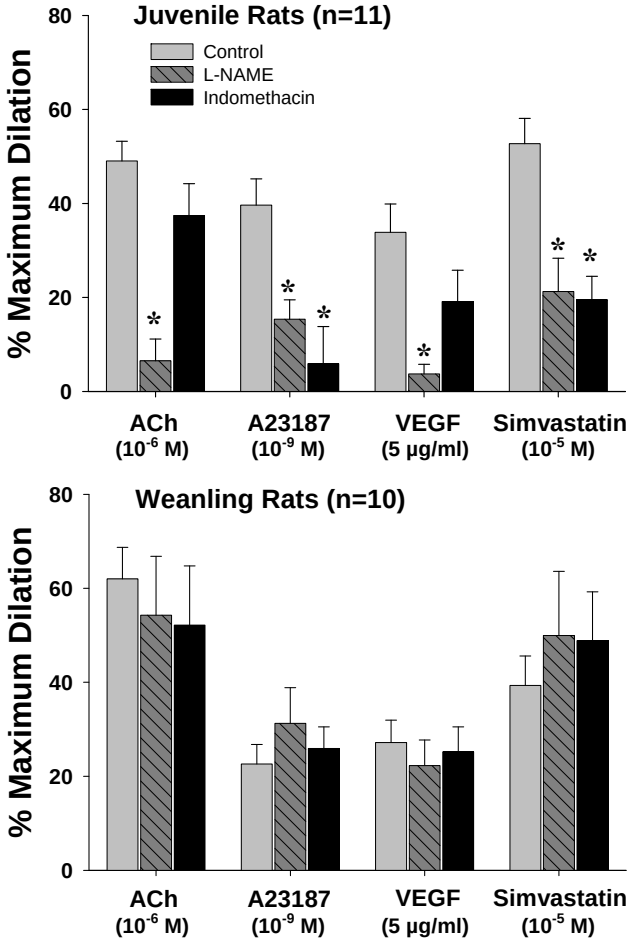
Responses of arterioles (in % maximum dilation) from weanling and juvenile rats to NS 1619 (30 μM) before and after inhibition of K_{ATP} channels with 10^{-6} M glibenclamide (Glib) or inhibition of K_{Ca} channels with 1 mM TEA, separately and in combination. Values are given as means ± SE. * $P<0.05$ vs. preceding response to NS 1619.

Figure 1: Responses of isolated gracilis muscle arterioles from weanling and juvenile rats to ACh, simvastatin, A23187 and VEGF



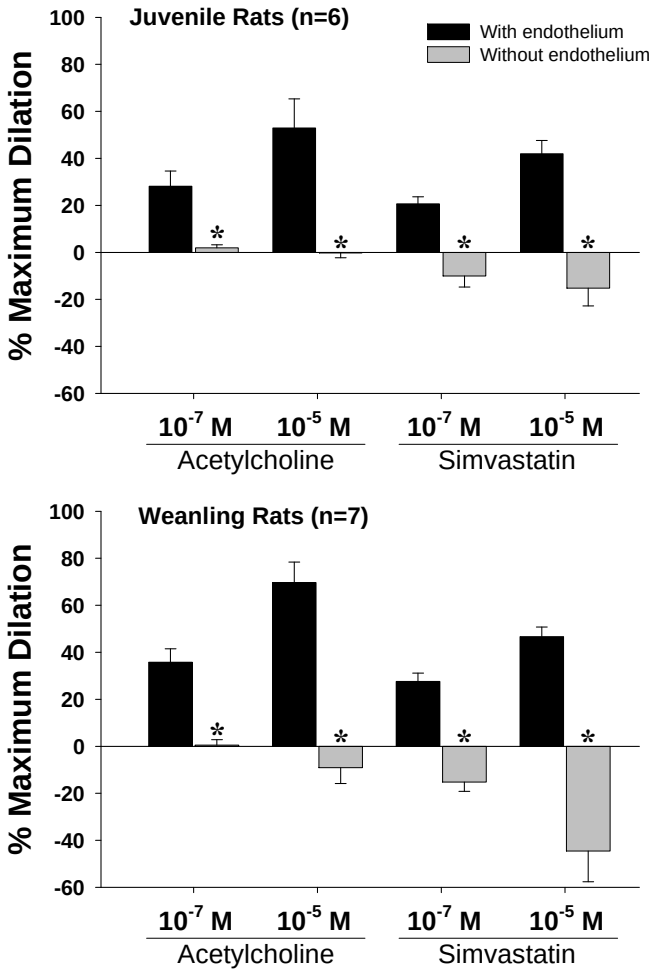
n = number of vessels. Values are given as means ± SE. *$p<0.05$ vs. Weanlings at same agonist concentration.

Figure 2: Responses of arterioles from juvenile and weanling rats to ACh, simvastatin, A23187 and VEGF after either L-NAME or indomethacin treatment



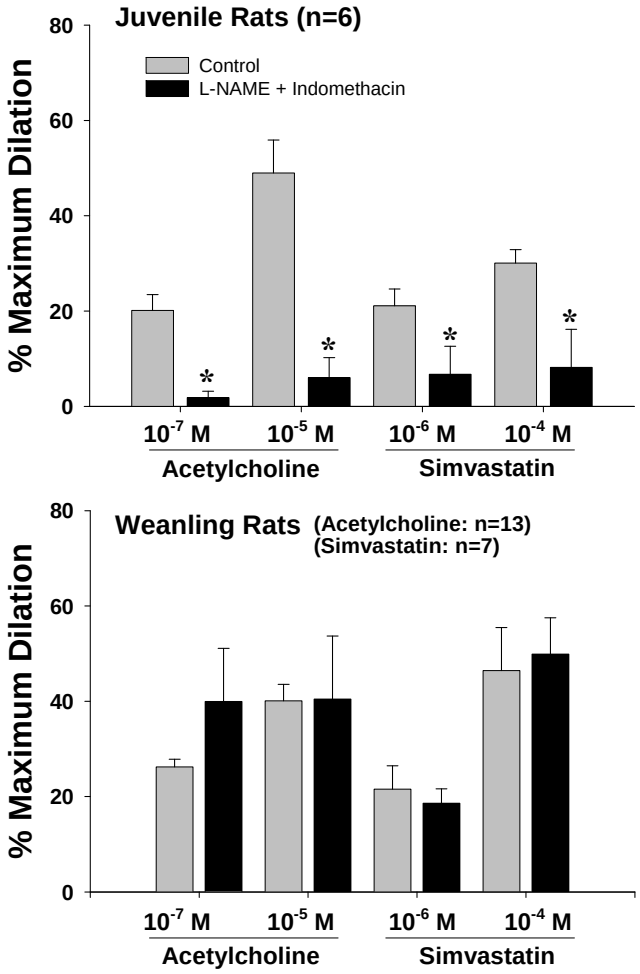
Data are presented under control conditions and following inhibition of NOS with L-NAME (10^{-4} M) and cyclooxygenase with indomethacin (10^{-6} M). n = number of vessels. Values are given as means ± SE. * $p<0.05$ vs. Control.

Figure 3: Effect of endothelial removal on responses of juvenile and weanling arterioles to ACh and simvastatin



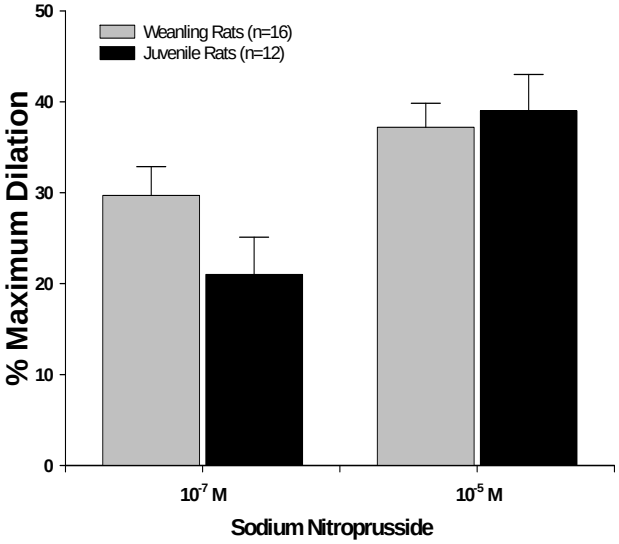
n=number of vessels. Values are given as means ± SE. * $p<0.05$ vs. vessel with intact endothelium.

Figure 4: Effect of combined NOS and COX inhibition on responses of juvenile and weanling arterioles to ACh and simvastatin



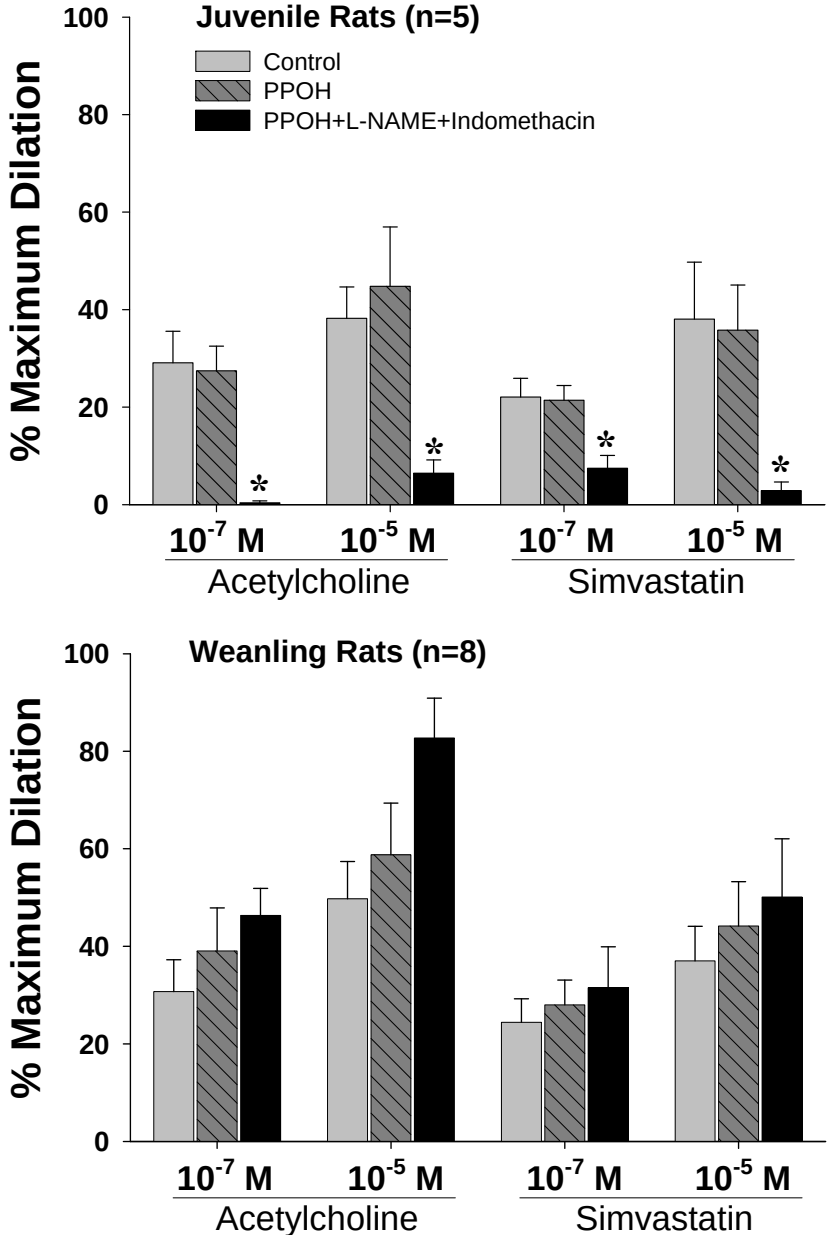
n = number of vessels. Values are given as means ± SE. * $p<0.05$ vs. Control.

Figure 5: Responses of arterioles from weanling and juvenile rats to SNP



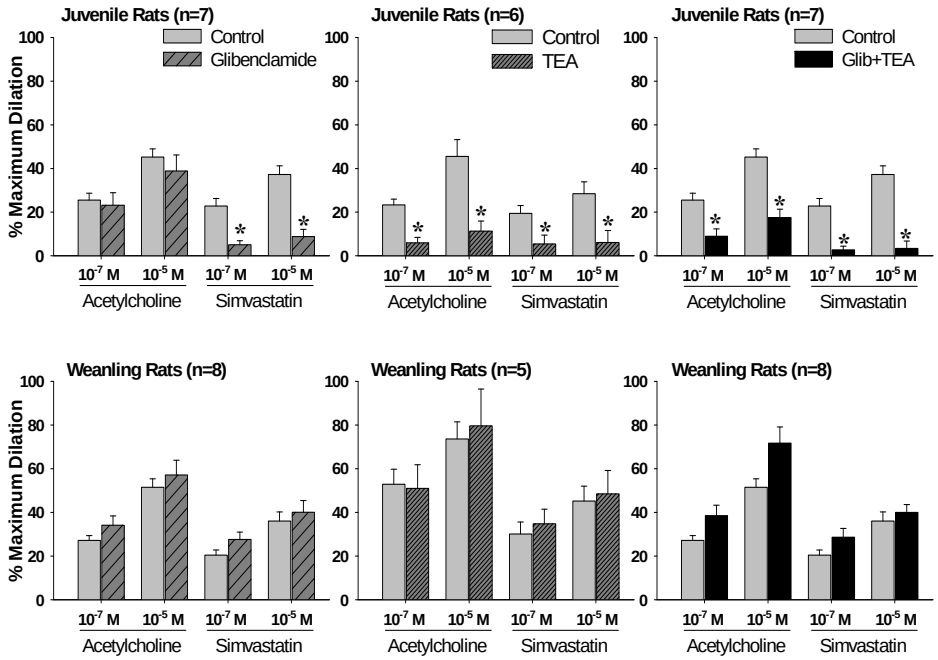
n = number of vessels. Values are given as means ± SE.

Figure 6: Responses of arterioles from juvenile and weanling rats to ACh and simvastatin before and after inhibition of cytochrome P-450 epoxygenases with PPOH, alone and in combination with NOS + COX inhibition.



n = number of vessels. Values are given as means ± SE. * $p<0.05$ vs. Control.

Figure 7: Responses of arterioles to ACh and simvastatin before and after K_{ATP} and K_{Ca} channel inhibition



Responses of arterioles from juvenile and weanling rats to ACh and simvastatin before and after inhibition of K_{ATP} channels with glibenclamide (left panels), inhibition of K_{Ca} channels with 1 mM TEA (center panels), and combined inhibition of K_{ATP} and K_{Ca} channels (right panels). n = number of vessels. Values are given as means ± SE. * $p<0.05$ vs. control.

Discussion

Growth-related changes in the responsiveness of conduit arteries to endothelium-dependent agonists have been well documented (DeMey & Gray, 1985; Koga *et al.*, 1989; Zellers & Vanhoutte, 1991; Koga *et al.*, 1992). Some investigators have reported varying degrees of such changes in endothelial function in the microcirculation (Nelson & Quayle, 1995; Linderman & Boegehold, 1999; Willis & Leffler, 2001), and the present study was designed to obtain further information on the endothelial factors that could control arteriolar tone at two different stages of juvenile growth. Our findings indicate that for juvenile arterioles, endothelium-dependent dilations to the agonists we used are mediated largely by NO, with vasodilator prostanoids also making an important contribution to these responses in some cases. However, metabolites of CP450 epoxygenase do not appear to contribute to any of the responses we investigated, although the clear role of potassium channels in these responses is consistent with the possibility that another type of EDHF is involved. In contrast, the endothelium-dependent dilation of weanling arterioles does not appear to rely on endothelial NO, prostanoids, CP450 epoxygenase products or any other EDHF.

BK-induced dilation of newborn porcine pial arterioles relies on prostaglandins, but becomes more reliant on NO release during juvenile growth (Willis & Leffler, 2001). Consistent with these observations, cyclooxygenase expression and activity is similar in newborn and adult porcine cerebral microvessels, whereas the adult microvessels have significantly higher NOS expression and activity (Parfenova *et al.*, 2000). From our current observations, it appears that NO, and in some cases cyclooxygenase products, contributes to the endothelium-dependent dilation of gracilis muscle arterioles from

juvenile, but not weanling rats (Figures 2 and 4). The absence of a role for NO in the endothelium-dependent dilation of weanling arterioles could theoretically be due to a lack of NO production, an accelerated breakdown of NO, or a reduced responsiveness of vascular smooth muscle to NO. The latter possibility appears unlikely, in light of the similar responsiveness to SNP in both age groups (Figure 5). This has been a consistent finding in other studies as well (Koga *et al.*, 1989; Zellers & Vanhoutte, 1991; Linderman & Boegehold, 1999; Nurkiewicz & Boegehold, 2004).

In adult animals, there is convincing evidence that endothelium-derived hyperpolarizing factors (EDHF) can contribute to endothelium-dependent arteriolar dilation (Rubanyi, 1993; Mombouli *et al.*, 1996; Shimokawa *et al.*, 1996). EDHF also appears to be an important mediator of vasodilation in the neonatal cerebral microcirculation (Lacza *et al.*, 2002). Although there is no single EDHF, metabolites of arachidonic acid by cytochrome P450 epoxygenase appear to play this role in rat cremaster muscle arterioles (McSherry *et al.*, 2006). To assess the potential role of CP450 4A-derived epoxyeicosatrienoic acids in the dilator responses we studied, the vessels were exposed to PPOH, a selective inhibitor of arachidonate epoxygenation reactions catalyzed by CP450 enzymes. PPOH had no effect on responses to ACh or simvastatin in either juvenile or weanling arterioles (Figure 6). In juvenile arterioles, the reduction of ACh- and simvastatin-induced dilation by L-NAME + indomethacin + PPOH was not different from that caused by L-NAME + indomethacin without PPOH (Figure 4). In weanling arterioles, addition of PPOH + L-NAME + indomethacin did not reduce responses to either ACh or simvastatin. Collectively, these observations indicate that CP450 epoxygenase metabolites do not contribute to the endothelium-dependent

dilation of gracilis muscle arterioles from juvenile or weanling rats, which is consistent with previous findings in gracilis muscle arterioles of older (12 week-old) rats (Ungvari & Koller, 2001).

It is possible that an EDHF derived from some other enzymatic pathway could have contributed to endothelium-dependent dilation in either or both of the age groups we studied. EDHFs generally exert their effects through activation of vascular smooth muscle K^+ channels (Welsh & Segal, 2000; Coleman *et al.*, 2001). Although some studies indicate that EDHFs only activate K_{Ca} channels (Ungvari & Koller, 2001), others have documented that K_{ATP} channels can also be activated by EDHFs (Brayden, 1990; Feletou & Vanhoutte, 1996). Therefore, we chose to target both of these channel types as potential mediators of dilation. The K_{ATP} channel inhibitor glibenclamide completely abolished simvastatin responses in juvenile arterioles, but had no effect on these responses in weanling arterioles, and had no effect on ACh responses in either juvenile or weanling arterioles (Figure 7). Although inhibition of K_{Ca} channels with 1mM TEA significantly reduced responses to both acetylcholine and simvastatin in juvenile arterioles (by 72-79%), it had no effect on these dilations in weanling arterioles. Inhibition of both K_{ATP} and K_{Ca} channels reduced responses to ACh by 60% - 70% and completely abolished responses to simvastatin in juvenile arterioles, but had no effect on the responses of weanling arterioles. We also used a high dose of TEA (5 mM) to block all K_{Ca} channels as well as other potential potassium channels in the weanling arterioles (Korn & Trapani, 2005), but only achieved a slight attenuation of the response to 10^{-5} M simvastatin (data not shown). Therefore, although juvenile arterioles apparently rely on

an EDHF to mediate dilations, we were not able to detect a contribution of EDHF to weanling arteriole dilations from our use of these potassium channel antagonists.

The K_{ATP} channel opener pinacidil dilated arterioles from rats of both age groups (Table 3), indicating the presence of recruitable K_{ATP} channels that are capable of modulating vascular smooth muscle tone. However, glibenclamide, at a concentration that inhibits most of this K_{ATP} channel activity, has no effect on the resting tone of arterioles from either group (Table 2). This suggests that if there is some level of K_{ATP} channel activity in these arterioles under our steady-state conditions, it is not sufficient to modulate smooth muscle contractile activity. In contrast, TEA, at a concentration that blocks the effect of a BK_{Ca} channel opener on arteriolar tone (Table 4), reduces the resting diameter of arterioles from both age groups by approximately the same amount (Table 2). This suggests that under resting conditions, there is ongoing arteriolar BK_{Ca} channel activity that is high enough to influence smooth muscle contractile activity, and that the magnitude of this effect does not appreciably change during rapid juvenile growth. This apparent influence of BK_{Ca} channel activity on gracilis muscle arteriolar tone is consistent with previous findings for this vessel (Ungvari & Koller, 2001), but these observations do not extend to arterioles in all skeletal muscles. Other investigators have found that smooth muscle K_{Ca} channel activity is "silent" in arterioles under resting conditions in both hamster and rat cremaster muscle (Jackson, 2005). Our interpretations based on the effects of TEA should be made with some caution; although TEA at a concentration of 1 mM will preferentially block K_{Ca} channels, the relatively broad specificity of this compound allows it to antagonize other types of potassium channels under some conditions (Fatherazi & Cook, 1991). This may have occurred to some

extent in the current study, as evidenced from our finding that 1 mM TEA did have some effect on the response of weanling arterioles to the K_{ATP} channel opener pinacidil (Table 3).

Consistent with the apparently similar influence of BK_{Ca} channels on resting tone in both age groups, the selective BK_{Ca} channel opener NS 1619 was as effective in dilating weanling arterioles as in dilating juvenile arterioles, with these responses being completely abolished by 1mM TEA in both age groups (Table 4). This is not consistent with previous reports that there are changes in K_{Ca} channel activity and density with maturation and growth. Teng et al (Teng *et al.*, 2002) found that stretch-induced activation of K_{Ca} channels exert a greater effect on resting vascular tone in cerebral arteries from adult sheep than in those from fetal sheep. Other investigators, using whole-cell patch-clamp recordings, determined that carotid body K_{Ca} channel expression increases with postnatal age in the rat (Hatton *et al.*, 1997). It is not clear why these differences exist, but perhaps some of the discrepancy can be explained by the use of different preparations and vessel size.

Direct microvascular pressure measurements indicate that *in situ*, lumenal pressure in the vessels we studied is equal to approximately 80% of mean arterial pressure (DeLano *et al.*, 1991). When equilibrated at this pressure *in vitro*, arterioles from weanling rats develop a significantly higher level of resting tone than arterioles from juvenile rats (Table 1), suggesting that arteriolar smooth muscle responsiveness to myogenic stimuli could be greater in the younger rats. However, a rigorous assessment of this possibility, which would require a determination of the different levels of myogenic tone developed over a wide range of intravascular pressures in each age group,

is beyond the scope of this study. Nevertheless, it may be germane to point out that over a wide range of lumenal pressures, the spontaneous tone developed by isolated small cerebral arteries from newborn mice is far greater than that developed by the same arteries from 6-8 week-old mice (Geary *et al.*, 2003). However, this is apparently not due to a greater myogenic responsiveness of smooth muscle in the younger vessels, but rather to less moderation of this myogenic tone by endothelial factors in these vessels. It is possible that such a differential effect of the endothelium could also account for the difference in tone between the two age groups studied here, but this seems unlikely because we found no evidence for any endothelial modulation of resting tone, even in mature vessels (i.e., no effect of L-NAME, indomethacin or PPOH on resting arteriolar diameters).

Using an *in vivo* exteriorized spinotrapezius muscle preparation, our laboratory previously found that Ca^{2+}-independent signaling pathways for endothelial NO release do not appear to be operational in arterioles of weanling rats (Nurkiewicz & Boegehold, 2004). However, in the current study on isolated gracilis muscle arterioles, we found no systemic age-related differences in responsiveness to agonists that promote either Ca^{2+}-dependent NO release (acetylcholine, A23187) or Ca^{2+}-independent NO release (simvastatin, VEGF), with the exception of the slightly smaller dilation of juvenile arterioles to one concentration of acetylcholine (Figure 1). This discrepancy may be due to the inherent heterogeneity between vascular beds.

As can be seen from Table 1, the period from 25 to 42 days of age is one of rapid juvenile growth in the rat, characterized by almost a 3-fold increase in body mass. The phases of human growth that correspond to these ages are somewhat imprecise because

they depend to some extent on the variables that are being compared. For example, if a comparison is made on the basis of projected weight gain, then our 25-day old and 42-day old rats, with average weights that are respectively 11% and 33% of an expected 550 g adult weight (from comparing Table 1 with Harlan Sprague Dawley growth tables), would roughly correspond to boys at the ages of 2 and 8 years (from CDC growth charts). However, if the comparison is made on the basis of current age relative to an average life expectancy of 728 days for the rat (Harlan Sprague Dawley) vs. 71.5 years for U.S. males (CDC), then these ages in the rat would roughly correspond to boys at 2 and 4 years of age. Finally, if the comparison is made on the basis of current age in relation to mean age of sexual maturity (57 days for rats; 13 years for human males), these ages in the rat would roughly correspond to boys at 6 and 10 years of age.

In conclusion, at any given time, the tone of skeletal muscle arterioles does not reflect a single mechanism, but rather an integrated effect of multiple mechanisms that, to some extent, may compensate for one another should one pathway be abolished. Endothelium-dependent arteriolar dilation in juvenile rats appears to be mediated by the combined release of endothelial nitric oxide and vasodilator prostanoids, with a non-CP450-derived hyperpolarizing factor possibly also contributing to the dilation. However, during earlier microvascular growth, this dilation is mediated by other factors yet to be identified.

References

Aquin L, Sillau AH, Lechner AJ & Banchero N. (1980). Growth and skeletal muscle microvascularity in the guinea pig. *Microvascular research* 20, 41-50.

Berg BR & Sarelius IH. (1996). Erythrocyte flux in capillary networks during maturation: implications for oxygen delivery. *The American journal of physiology* 271, H2263-2273.

Brayden JE. (1990). Membrane hyperpolarization is a mechanism of endothelium-dependent cerebral vasodilation. *The American journal of physiology* 259, H668-673.

Coleman HA, Tare M & Parkington HC. (2001). EDHF is not K^+ but may be due to spread of current from the endothelium in guinea pig arterioles. *The American journal of physiology* 280, H2478-2483.

DeLano FA, Schmid-Schonbein GW, Skalak TC & Zweifach BW. (1991). Penetration of the systemic blood pressure into the microvasculature of rat skeletal muscle. *Microvascular research* 41, 92-110.

DeMey J & Gray S. (1985). Endothelium-dependent reactivity in resistance vessels. *Prog appl microcirc* 8, 181-187.

Fallet RW, Bast JP, Fujiwara K, Ishii N, Sansom SC & Carmines PK. (2001). Influence of Ca^{2+}-activated K^+ channels on rat renal arteriolar responses to depolarizing agonists. *Am J Physiol Renal Physiol* 280, F583-591.

Fatherazi S & Cook DL. (1991). Specificity of tetraethylammonium and quinine for three K^+ channels in insulin-secreting cells. *The Journal of membrane biology* 120, 105-114.

Feletou M & Vanhoutte PM. (1996). Endothelium-derived hyperpolarizing factor. *Clinical and experimental pharmacology & physiology* 23, 1082-1090.

Fredricks KT, Liu Y & Lombard JH. (1994a). Response of extraparenchymal resistance arteries of rat skeletal muscle to reduced PO_2. *The American journal of physiology* 267, H706-715.

Fredricks KT, Liu Y, Rusch NJ & Lombard JH. (1994b). Role of endothelium and arterial K^+ channels in mediating hypoxic dilation of middle cerebral arteries. *The American journal of physiology* 267, H580-586.

Frisbee JC, Roman RJ, Murali Krishna U, Falck JR & Lombard JH. (2001). Altered mechanisms underlying hypoxic dilation of skeletal muscle resistance arteries of hypertensive versus normotensive Dahl rats. *Microcirculation* 8, 115-127.

Geary GG, Buchholz JN & Pearce WJ. (2003). Maturation depresses mouse cerebrovascular tone through endothelium-dependent mechanisms. *Am J Physiol Regul Integr Comp Physiol* 284, R734-741.

Hatton CJ, Carpenter E, Pepper DR, Kumar P & Peers C. (1997). Developmental changes in isolated rat type I carotid body cell K^+ currents and their modulation by hypoxia. *The Journal of physiology* 501 (Pt 1), 49-58.

Jackson WF. (2005). Potassium channels in the peripheral microcirculation. *Microcirculation* 12, 113-127.

Koga T, Takata Y, Kobayashi K, Fujii K, Nagao T & Fujishima M. (1992). Age-related changes in P_2-purinergic receptors on vascular smooth muscle and endothelium. *Hypertension* 19, 286-289.

Koga T, Takata Y, Kobayashi K, Takishita S, Yamashita Y & Fujishima M. (1989). Age and hypertension promote endothelium-dependent contractions to acetylcholine in the aorta of the rat. *Hypertension* 14, 542-548.

Korn SJ & Trapani JG. (2005). Potassium channels. *IEEE transactions on nanobioscience* 4, 21-33.

Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, Lefer DJ, Sessa WC & Walsh K. (2000). The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. *Nature medicine* 6, 1004-1010.

Lacza Z, Puskar M, Kis B, Perciaccante JV, Miller AW & Busija DW. (2002). Hydrogen peroxide acts as an EDHF in the piglet pial vasculature in response to bradykinin. *The American journal of physiology* 283, H406-411.

Linderman JR & Boegehold MA. (1996). Arteriolar network growth in rat striated muscle during juvenile maturation. *International journal of microcirculation, clinical and experimental / sponsored by the European Society for Microcirculation* 16, 232-239.

Linderman JR & Boegehold MA. (1999). Growth-related changes in the influence of nitric oxide on arteriolar tone. *The American journal of physiology* 277, H1570-1578.

Lombard JH, Liu Y, Fredricks KT, Bizub DM, Roman RJ & Rusch NJ. (1999). Electrical and mechanical responses of rat middle cerebral arteries to reduced PO_2 and prostacyclin. *The American journal of physiology* 276, H509-516.

Mabanta L, Valane P, Borne J & Frame MD. (2006). Initiation of remote microvascular preconditioning requires K_{ATP} channel activity. *The American journal of physiology* 290, H264-271.

McSherry IN, Sandow SL, Campbell WB, Falck JR, Hill MA & Dora KA. (2006). A role for heterocellular coupling and EETs in dilation of rat cremaster arteries. *Microcirculation* 13, 119-130.

Mombouli JV, Bissiriou I, Agboton V & Vanhoutte PM. (1996). Endothelium-derived hyperpolarizing factor: a key mediator of the vasodilator action of bradykinin. *Immunopharmacology* 33, 46-50.

Nelson MT & Quayle JM. (1995). Physiological roles and properties of potassium channels in arterial smooth muscle. *The American journal of physiology* 268, C799-822.

Nurkiewicz TR & Boegehold MA. (2004). Calcium-independent release of endothelial nitric oxide in the arteriolar network: onset during rapid juvenile growth. *Microcirculation* 11, 453-462.

Olesen SP, Munch E, Moldt P & Drejer J. (1994). Selective activation of Ca^{2+}-dependent K^+ channels by novel benzimidazolone. *European journal of pharmacology* 251, 53-59.

Parfenova H, Massie V & Leffler CW. (2000). Developmental changes in endothelium-derived vasorelaxant factors in cerebral circulation. *The American journal of physiology* 278, H780-788.

Rubanyi GM. (1993). The role of endothelium in cardiovascular homeostasis and diseases. *Journal of cardiovascular pharmacology* 22 Suppl 4, S1-14.

Sarelius IH, Damon DN & Duling BR. (1981). Microvascular adaptations during maturation of striated muscle. *The American journal of physiology* 241, H317-324.

Shimokawa H, Yasutake H, Fujii K, Owada MK, Nakaike R, Fukumoto Y, Takayanagi T, Nagao T, Egashira K, Fujishima M & Takeshita A. (1996). The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *Journal of cardiovascular pharmacology* 28, 703-711.

Standen NB, Quayle JM, Davies NW, Brayden JE, Huang Y & Nelson MT. (1989). Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science* 245, 177-180.

Teng GQ, Nauli SM, Brayden JE & Pearce WJ. (2002). Maturation alters the contribution of potassium channels to resting and 5HT-induced tone in small cerebral arteries of the sheep. *Brain research* 133, 81-91.

Uluoglu C & Zengil H. (2003). Comparison of different de-endothelization procedures in the isolated rat thoracic aorta: a short communication. *Research communications in molecular pathology and pharmacology* 113-114, 289-297.

Ungvari Z & Koller A. (2001). Mediation of EDHF-induced reduction of smooth muscle $[Ca^{2+}]_i$ and arteriolar dilation by K^+ channels, 5,6-EET, and gap junctions. *Microcirculation* 8, 265-274.

Ungvari Z, Sun D, Huang A, Kaley G & Koller A. (2001). Role of endothelial $[Ca^{2+}]$i in activation of eNOS in pressurized arterioles by agonists and wall shear stress. *The American journal of physiology* 281, H606-612.

Wang DH & Prewitt RL. (1991). Microvascular development during normal growth and reduced blood flow: introduction of a new model. *The American journal of physiology* 260, H1966-1972.

Wang MH, Brand-Schieber E, Zand BA, Nguyen X, Falck JR, Balu N & Schwartzman ML. (1998). Cytochrome P450-derived arachidonic acid metabolism in the rat kidney: characterization of selective inhibitors. *The Journal of pharmacology and experimental therapeutics* 284, 966-973.

Welsh DG & Segal SS. (2000). Role of EDHF in conduction of vasodilation along hamster cheek pouch arterioles in vivo. *The American journal of physiology* 278, H1832-1839.

Willis AP & Leffler CW. (2001). Endothelial NO and prostanoid involvement in newborn and juvenile pig pial arteriolar vasomotor responses. *The American journal of physiology* 281, H2366-2377.

Zellers TM & Vanhoutte PM. (1991). Endothelium-dependent relaxations of piglet pulmonary arteries augment with maturation. *Pediatric research* 30, 176-180.

Zweifach BW, Kovalcheck S, De Lano F & Chen P. (1981). Micropressure-flow relationships in a skeletal muscle of spontaneously hypertensive rats. *Hypertension* 3, 601-610.

III. STUDY 2: Hydrogen Peroxide Emerges as a Regulator of Arteriolar Tone in Skeletal Muscle during Juvenile Growth

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Abstract

Endothelium-dependent dilation of skeletal muscle arterioles is mediated by factors that are yet to be identified in young rats, and partly mediated by at least one unidentified hyperpolarizing factor in older juvenile rats. We assessed the possible contribution of hydrogen peroxide (H_2O_2) to this arteriolar dilation at both of these growth stages. Gracilis muscle arterioles were isolated from rats at ages 24-26 days (weanlings) and 46-48 days (juveniles). Simvastatin and ACh induced endothelium-dependent dilations that were attenuated by catalase in juvenile, but not weanling, arterioles. Juvenile, but not weanling, arterioles also displayed catalase-sensitive 2′,7′ dichlorofluorescein fluorescence that was increased by ACh. In juvenile arterioles, exogenous H_2O_2 induced constriction at low concentrations and dilation at higher concentrations. In weanling arterioles, H_2O_2 induced only constriction or had no effect. Responses to low and high concentrations of H_2O_2 were attenuated by NOS inhibition in juvenile, but not weanling, arterioles, but equally attenuated by endothelial removal in both groups. In intact weanling and juvenile arterioles, the K^+ channel inhibitors TEA and glibenclamide abolished constrictions to lower levels of H_2O_2, and unmasked constrictor responses to higher levels of H_2O_2. These findings suggest that endogenous H_2O_2 contributes to endothelium-dependent arteriolar dilation in juvenile rats but not in

younger rats, and that this H_2O_2 acts in juvenile rats partly by stimulating endothelial NO release and activating K^+ channels.

Introduction

Although increased levels of hydrogen peroxide (H_2O_2) are sometimes responsible for the vascular dysfunction associated with certain disease states (Lum & Roebuck, 2001; Cai, 2005), H_2O_2 at lower concentrations has also been recognized as an important signaling molecule that can mediate normal vascular function (Kunsch & Medford, 1999; Chen *et al.*, 2003). For example, endothelium-derived H_2O_2 has been identified as one of the factors that mediate arteriolar dilation to some agonists in the cerebral (Sobey *et al.*, 1997), mesenteric (Matoba *et al.*, 2000) and coronary (Matoba *et al.*, 2003, Oltman *et al.*,, 2003) vascular beds, and there is mounting evidence for a true physiological role of arteriolar H_2O_2 in local coronary blood flow regulation (Miura *et al.*, 2003; Yada *et al.*, 2003; Koller & Bagi, 2004). Our laboratory has recently reported that endogenous H_2O_2 can also contribute to the arteriolar dilation and hyperemia that accompanies skeletal muscle contraction (Marvar & Boegehold, 2006). However, in that study we were not able to determine the extent to which this H_2O_2 originates from the arteriolar wall versus the surrounding muscle fibers. In fact, there is little information on whether arterioles in skeletal muscle are intrinsically capable of releasing vasoactive amounts of H_2O_2 in response to any stimulus.

Other studies from our laboratory have documented that postnatal growth of the microvasculature in skeletal muscle is accompanied by progressive changes in a number of factors that can influence arteriolar tone and blood flow (Linderman & Boegehold,

1996; Linderman & Boegehold, 1999; Lum & Roebuck, 2001; Balch Samora *et al.*, 2007). For example, skeletal muscle arterioles from young rats exhibit endothelium-dependent dilation, but these responses are not mediated by either nitric oxide (NO) or cyclooxygenase metabolites, as they are in arterioles from mature rats (Balch Samora *et al.*, 2007). The endothelial factors that mediate this dilation in young arterioles have not been identified, but because H_2O_2 can serve as an important endothelium-derived vasoactive factor in the cerebral microcirculation of newborn pigs (Lacza *et al.*, 2002), we undertook the current study to determine if H_2O_2 also mediates endothelium-dependent dilation in skeletal muscle arterioles of young rats. Arterioles from the gracilis muscle of rapidly-growing rats at two different ages were isolated and studied in vitro. A second aim of this study was to determine if there are any age-related differences in the mechanism by which H_2O_2 can influence arteriolar smooth muscle tone.

Materials and Methods

Animals: All surgical and experimental procedures were approved by the West Virginia University Animal Care and Use Committee. Experiments were performed on gracilis muscle arterioles isolated from male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) at 3-4 weeks of age ("weanlings") or 6-7 weeks of age ("juveniles").

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight), with supplemental anesthetic (10% of original dose) administered if needed. The right carotid artery was cannulated with polyethylene tubing (PE-10 for

weanlings, PE-50 for juveniles) to measure mean arterial pressure before removal of the gracilis arteriole.

Preparation of Isolated Vessels: An arteriolar branch of the femoral artery supplying the gracilis muscle was removed, handling only the surrounding connective tissue to minimize vessel stretching or damage. The rat was sacrificed by intracardiac injection of sodium pentobarbital immediately after vessel removal. The vessel was placed in warmed physiological salt solution (PSS) equilibrated with 21% O_2, 5% CO_2, and 74% N_2, and having the following composition (mM): 119 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 1.6 $CaCl_2$, 1.18 NaH_2PO_4, 24 $NaHCO_3$, 0.026 EDTA, and 5.5 glucose. After isolation, each vessel was prepared for in vitro video microscopy as previously described (Fredricks, Liu & Lombard, 1994). Briefly, the vessel was mounted in a heated (37º C) chamber that allowed for its lumen and exterior surface to be perfused and superfused, respectively, with PSS from separate reservoirs. The vessel was cannulated at both ends with glass micropipettes (50- and 70-μm tip diameters for weanling and juvenile vessels, respectively) and secured to the inflow and outflow pipettes using 9-0 nylon suture. Any side branches were ligated with a single strand teased from 6-0 silk suture. The inflow pipette was connected to a reservoir perfusion system for control of intralumenal pressure and flow. The vessel was then extended to its in situ length and equilibrated at 80% of the animal's mean arterial pressure to approximate its normal in vivo perfusion pressure (DeLano *et al.*, 1991).

Vessel diameter was measured using an onscreen video micrometer. Any vessel that did not demonstrate endothelial viability, as judged by at least a 10% dilation in response to 10-7 M acetylcholine (ACh, Sigma Chemical, St. Louis, MO), was not used

in the study. Using this criterion, approximately 1 in 10 vessels from each age group were discarded. In such cases, we then isolated and studied the identical arteriole from the animal's contralateral gracilis muscle. As a result, we were able to obtain a viable arteriole from each animal. Diameter measurements were made under static, zero-flow conditions after a 30-minute equilibration period with continuous perfusion. Resting vascular tone was calculated as $(\Delta D/D_{max}) \times 100$, where ΔD is the diameter increase from rest in response to Ca^{2+}-free PSS (30-40 minute equilibration with Ca^{2+}-free bath solution and no lumenal flow), and D_{max} is the maximum diameter measured under these conditions.

Agonists: ACh, at bath concentrations of 10^{-5} or 10^{-7} M, was used to assess arteriolar capacity for Ca^{2+}-dependent endothelial NO formation (Ungvari *et al.*, 2001). Simvastatin (Merck Research Laboratories, Rathway, NJ) was used to assess arteriolar capacity for Ca^{2+}-independent endothelial NO formation (Nurkiewicz & Boegehold, 2004). Simvastatin was activated by alkaline lysis (5.25 ml of 0.1 N NaOH per 140 mg, dissolved in 3.5 ml of ETOH) at 50ºC for 2 h. The resulting solution was then diluted to a volume of 35 ml with PBS, and neutralized to pH 7.4 with HCl. One-ml aliquots of this solution were then serially diluted with PBS, producing a final bath concentration of 10^{-5} or 10^{-7} M. The two concentrations of each agonist were applied in random order, separated by a washout period to allow the vessel to regain resting tone.

For exogenous H_2O_2 application, a 3% H_2O_2 aqueous solution was diluted with PSS to concentrations of $1x10^{-6}$, $5x10^{-6}$, $1x10^{-5}$, $5x10^{-5}$, and $1x10^{-4}$ M, and these increasing concentrations were sequentially added to the bath. After each application, the

H_2O_2 was washed out of the chamber with PSS, and the vessel was allowed to return to its control diameter before the next H_2O_2 application.

Assessment of Arteriolar H_2O_2 Production: 2′,7′ dichlorodihydrofluorescein diacetate (DCFH-DA) was used to detect intracellular H_2O_2 production (Miura *et al.*, 2003; Silveira *et al.*, 2003) in arterioles at rest or during exposure to 10^{-5} M ACh, either with or without catalase (Sigma, 80 U/ml, from bovine liver) present in the bath. To ensure maximal scavenging of H_2O_2, this concentration of catalase was higher than the concentration we have previously found to scavenge all H_2O_2 formed during arteriolar superoxide (O_2^-) generation in vivo (Lenda *et al.*, 2000).

Non-fluorescent DCFH-DA freely enters the cell, where it is de-acetated to DCFH and then oxidized by H_2O_2 to form fluorescent 2′,7′ dichlorofluorescein (DCF) (Tarpey & Fridovich, 2001). Under some conditions, DCFH can also be oxidized in vivo by cellular peroxidases, peroxynitrite, or even hypochlorous acid (Tarpey & Fridovich, 2001; Silveira *et al.*, 2003). Therefore, we considered only the portion of DCF fluorescence that could be reduced by catalase as an indicator of H_2O_2 activity.

After the arteriole was isolated, cannulated and allowed to develop spontaneous tone at 80% of mean arterial pressure, $5x10^{-5}$ M DCFH-DA was added to the vessel bath under one of the conditions described above. After 20 minutes of exposure to DCFH-DA, the arteriole was quickly removed from the glass micropipettes and transferred to the stage of a fluorescence microscope coupled to a high-sensitivity black and white CCD video camera (Dage-MTI, Michigan City, IN), and then epi-illuminated for 1 second using a mercury lamp and the appropriate excitation and emission filters for detection of DCF fluorescence (460-490 nm excitation, 515-550 nm barrier). In all cases, an

instantaneous image of the vessel was obtained immediately upon opening of the illumination diaphragm, with identical illumination settings and optical parameters used for each vessel. Metamorph software (v. 6.01, Universal Imaging, Downingtown PA) was used to acquire, digitize and store images for subsequent analysis (Zhu *et al.*, 2004). Average fluorescence intensity was determined for the entire vessel wall (*V*) as well as for the background (*B*) of each vessel. Fluorescence intensity, based on the standard 0-255 gray scale (Klabunde & Anderson, 2002), was quantified using the following equation:

$$\%I_{MAX} = V - B \big/ 255 - B$$

where $\%I_{MAX}$ is percent of maximum fluorescence intensity, *V* is the intensity of the vessel wall, and *B* is the background.

Scavengers and Inhibitors: To assess the role of reactive oxygen species in vessel responses, vessels were pretreated with catalase (see above) or the membrane-permeable superoxide dismutase (SOD) mimetic 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (tempol) (Thiemermann, 2003) (Sigma, 10^{-4} M), alone or in combination. These agents were added to the bath for 30 minutes before vessels were challenged with agonists. As a control for active catalase, the effects of denatured catalase on arteriolar responses to ACh and H_2O_2 were also assessed. For denaturization, catalase was boiled for 10 minutes. To determine the contribution of endothelial NO production to vessel responses, the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) was added to the bath at a concentration of 10^{-4} M for 20 minutes before agonist challenge (Frisbee *et al.*, 2001).

Since many endothelium-derived relaxing factors reduce arteriolar tone at least in part through activation of smooth muscle potassium (K^+) channels (Welsh & Segal, 2000; Coleman *et al.*, 2001; Ungvari *et al.*, 2002), the contribution of these channels to arteriolar dilation was assessed by using tetraethylammonium (TEA, Sigma) (Fredricks *et al.*, 1994; Lombard *et al.*, 1999; Fallet *et al.*, 2001), and the antidiabetic sulphonylurea, glibenclamide (Sigma) (Lombard *et al.*, 1999), in both intact and endothelium-denuded vessels (see below). We used a 10^{-3} M bath concentration of TEA to selectively block Ca^{2+}-activated K^+ (K_{Ca}) channels (Nelson & Quayle, 1995), and 10^{-6} M glibenclamide to selectively block K_{ATP} channels (Standen *et al.*, 1989).

Endothelial Denudation: To determine the role of the endothelium in mediating arteriolar responses to exogenous H_2O_2, the endothelium was removed in some experiments by mechanical abrasion (Uluoglu & Zengil, 2003). The pipette tip at each end of the vessel was gently advanced through the vessel lumen at least three times to ensure elimination of the endothelium. We have previously verified that this method successfully denudes the endothelium of gracilis muscle arterioles without affecting the underlying smooth muscle (Balch Samora, 2007).

All data are presented as mean ± SE. For all analyses, a probability value of $p < 0.05$ was considered to be statistically significant. Dilation in response to Ca^{2+}-free PSS is expressed as percent increase from control diameter. Differences between the means of individual experimental groups were determined by ANOVA/Newman-Keuls test, or by an unpaired Student's *t*-test when two independent means were compared.

Results

General characteristics of all rats from which vessels were removed for functional studies are reported in Table 1. Age, body weight, and mean arterial pressure were significantly greater in juvenile rats than in weanling rats. Table 1 also summarizes the general characteristics of all arterioles utilized for functional studies. Resting and passive diameters of the arterioles from juvenile rats were significantly greater than those of the arterioles from weanling rats, whereas there was no difference between groups in the level of spontaneous tone (resting vascular tone) that developed with vessel pressurization.

There were no differences between age groups in overall arteriolar responsiveness to ACh or simvastatin (Figure 1). Treatment with catalase, tempol, or catalase + tempol had no effect on the resting diameter of either weanling or juvenile arterioles (Table 2). However, catalase dramatically reduced responses to ACh and simvastatin in juvenile arterioles, but not in weanling arterioles (Figure 2, left panels). Denatured catalase had no effect on responses to ACh in either group (Table 3), verifying that the effects of catalase on juvenile arterioles were specifically due to the enzymatic activity of the molecule. Tempol by itself was without effect on responses to ACh or simvastatin in either group. In the juvenile arterioles, combined treatment with both catalase and tempol reduced ACh and simvastatin responses to levels that were not different from those with catalase treatment alone (top right panel). In weanling arterioles, catalase + tempol had no effect on responses to either agonist (bottom right panel).

A total of 26 juvenile arterioles and 22 weanling arterioles were examined after DCFH-DA exposure for determination of relative H_2O_2 levels. Compared to their respective control levels, 10^{-5} M ACh significantly increased DCF fluorescence in juvenile

arterioles (from 29 ± 4 to 44 ± 4% of maximum intensity), but not in weanling arterioles (Figure 3). Catalase reduced DCF fluorescence under control conditions as well as during ACh exposure in juvenile arterioles, but had no significant effect in weanling arterioles.

Application of exogenous H_2O_2 at concentrations ranging between $1x10^{-6}$ and $1x10^{-4}$ M elicited concentration-dependent responses from juvenile arterioles, with the lower concentrations causing constriction and the higher concentrations causing dilation (Figure 4). In 44% of these vessels, the dilation to higher concentrations of H_2O_2 was preceded by a transient constriction. Weanling arterioles also constricted in response to H_2O_2 at the lower concentrations, and these constrictions were significantly greater than those of the juvenile arterioles. However, whereas juvenile arterioles clearly dilated in response to higher concentrations of H_2O_2, weanling vessels did not.

Treatment with L-NAME had no significant effect on the resting diameters of either juvenile arterioles (51.3 ± 4.4 μm before vs. 48.1 ± 5.7 μm after L-NAME) or weanling arterioles (41.8 ± 3.4 μm vs. 39.3 ± 2.8 μm). However, L-NAME significantly attenuated the constriction of juvenile arterioles to H_2O_2 at the lowest concentration (10^{-6} M), and reduced the dilations of these vessels in response to higher concentrations of H_2O_2 (Figure 5, top panel). In contrast, L-NAME had no significant effect on weanling arteriolar responses to H_2O_2 at any concentration (bottom panel). Subsequent addition of catalase to the bath completely abolished the residual L-NAME-insensitive dilation of juvenile arterioles (top panel), and abolished the constriction to $1x10^{-6}$ M and $5x10^{-6}$ M H_2O_2 in weanling arterioles (bottom panel).

Treatment with TEA + glibenclamide significantly reduced the resting diameters of juvenile arterioles (50.9 ± 4.7 μm before vs. 35.9 ± 3.3 μm after treatment) and weanling

arterioles (35.9 ± 3.3 μm vs. 29.7 ± 1.5 μm). When expressed as percent reduction from control diameter, the magnitude of this effect was similar in the two groups (25-29%). For juvenile arterioles, TEA + glibenclamide abolished constrictor responses to $1x10^{-6}$ and $5x10^{-6}$ M H_2O_2, and converted the dilator responses to higher concentrations of H_2O_2 into constrictor responses (Figure 6, top panel). For weanling arterioles, TEA + glibenclamide converted the constrictor responses to lower concentrations of H_2O_2 into dilator responses but led to constrictor responses to H_2O_2 at higher concentrations (Figure 6, bottom panel). Both weanling and juvenile arterioles constricted to the two higher doses of H_2O_2 when pretreated with TEA + glibenclamide, with the constriction of weanling arterioles being significantly greater than that of juvenile arterioles.

Endothelial removal tended to reduce the resting diameters of juvenile arterioles (53.7 ± 2.3 μm before vs. 47.3 ± 4.2 μm after de-endothelialization) and weanling arterioles (35.9 ± 2.7 μm vs. 29.1 ± 1.8 μm), but these reductions did not reach statistical significance. However, removal of the endothelium significantly reduced the constrictor responses to $1x10^{-6}$ M and $5x10^{-6}$ M H_2O_2 in both juvenile and weanling arterioles, as well as constrictor responses to $1x10^{-5}$ M H_2O_2 in weanling arterioles (Figure 7). The dilation of juvenile arterioles to the higher concentrations of H_2O_2 ($5x10^{-5}$, $1x10^{-4}$ M) was also significantly attenuated by endothelial removal. Treatment of the de-endothelialized vessels with TEA + glibenclamide further reduced the dilator responses to H_2O_2 in juvenile arterioles, but had no further effect on the constrictor responses in either group.

Table 1: General characteristics of all rats and vessels used for functional studies

Animal Characteristics	**Weanlings**	**Juveniles**
N	42	39
Age (days)	25.2 ± 0.3	46.3 ± 0.8 *
Body Weight (g)	57.6 ± 1.2	170.4 ± 3.3 *
MAP (mmHg)	79.5 ± 1.9	96.4 ± 2.1 *
Vessel Characteristics	**Weanlings**	**Juveniles**
n	42	39
Resting Diameter (µm)	39.1 ± 1.4	55.9 ± 2.0 *
Passive Diameter (µm)	58.2 ± 1.8	82.6 ± 1.7 *
Resting Vascular Tone (%)	32.2 ± 2.0	32.3 ± 1.9

MAP = mean arterial pressure. Values are given as means ± SE. * $P<0.05$ vs. weanling group.

Table 2: Summary of resting vessel diameters under control conditions and after treatment with catalase and tempol, separately and in combination

	Arteriolar Diameter (μm)					
	Control	**Catalase (80 U/ml)**	**Control**	**Tempol (10^{-4} M)**	**Control**	**Catalase + Tempol**
Weanlings	45.4 ± 3.9	42.7 ± 4.5	45.4 ± 3.9	42.9 ±4.6	43.2 ± 2.6	43.4 ± 4.2
Juveniles	64.0 ± 4.0	62.4 ± 4.8	64.0 ± 4.0	58.2 ± 4.5	63.8 ± 3.8	59.9 ± 6.1

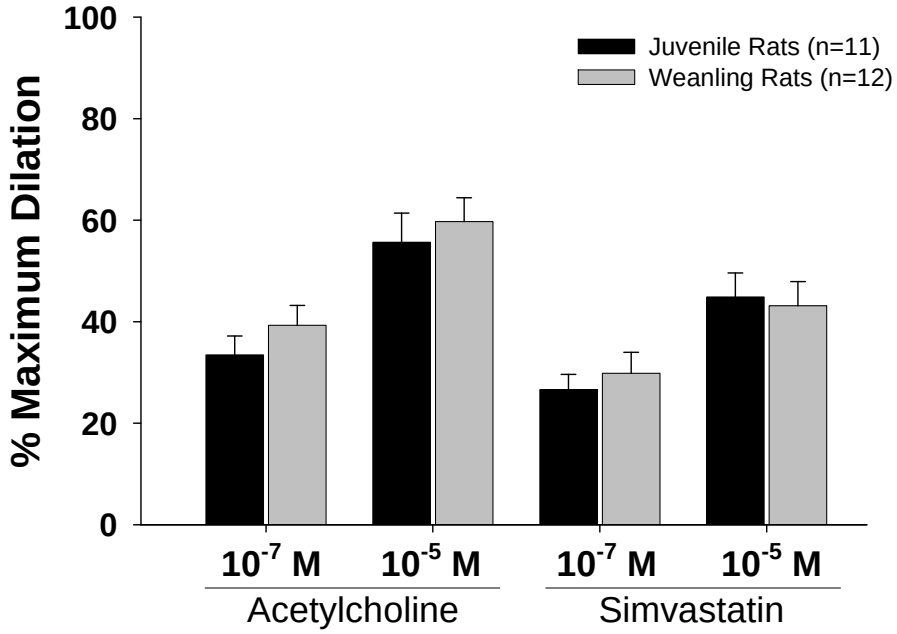
Values are given as means ± SE.

Table 3: Magnitude of arteriolar diameter changes in response to ACh and H_2O_2 before and after treatment with denatured catalase

	Weanlings (n=3)		**Juveniles (n=3)**	
	Control	Inactivated catalase	Control	Inactivated catalase
10^{-5} M ACh	32.3 ± 3.6	41.2 ± 7.9	32.8 ± 2.8	39.7 ± 7.6
10^{-6} M H_2O_2	-22.5 ± 10.4	-22.4 ± 9.4	-13.4 ± 2.7	-11.7 ± 5.0
10^{-5} M H_2O_2	-22.4 ± 9.4	-26.6 ± 11.1	-0.69 ± 4.1	-2.3 ± 1.1
10^{-4} M H_2O_2	-7.2 ± 3.9	-3.0 ± 3.0	16.8 ± 3.8	18.5 ± 8.0

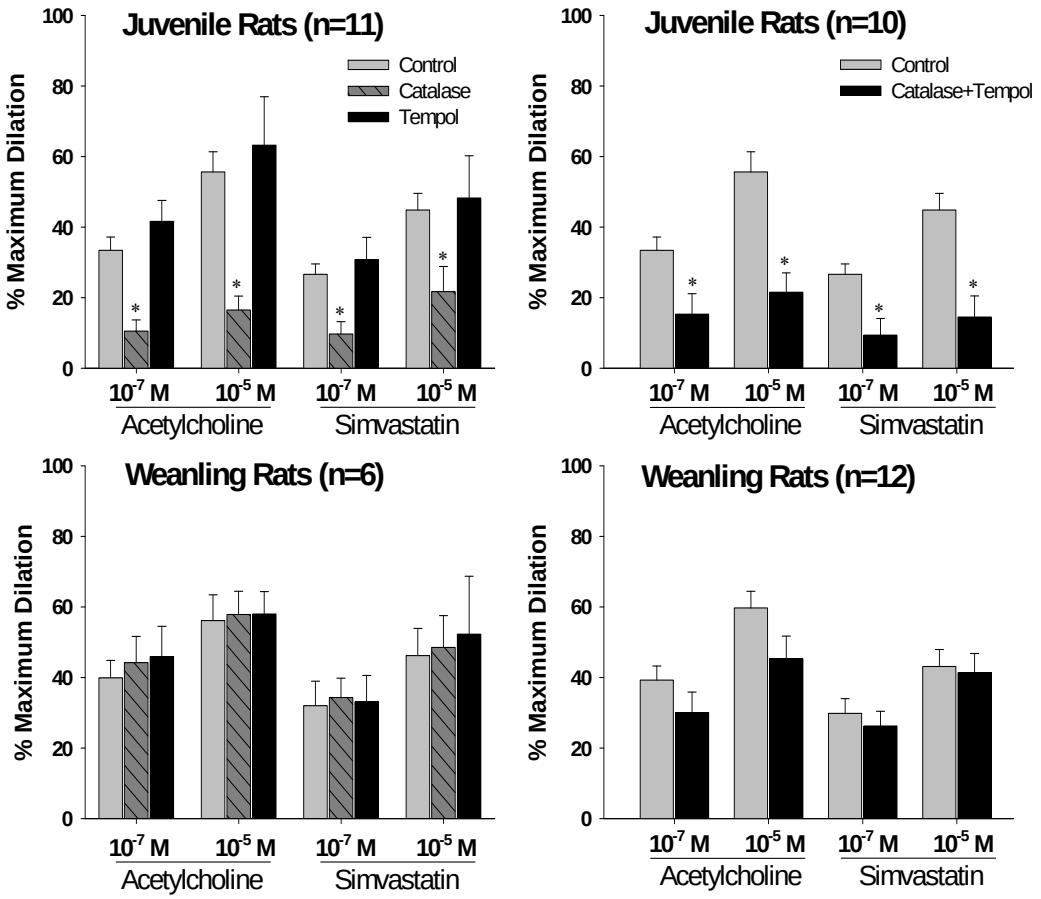
Values are given as means (% maximum dilation) ± SE. n = number of vessels.

Figure 1: Responses of gracilis muscle arterioles from juvenile and weanling rats to ACh and simvastatin



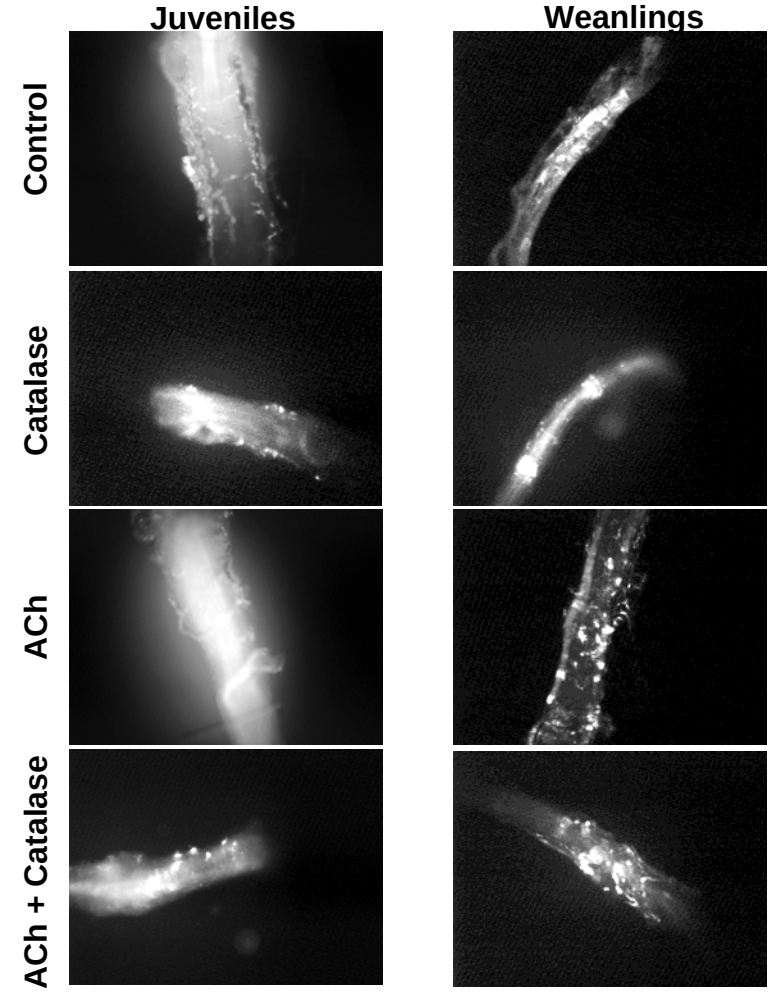
n = number of vessels. Values are given as means ± SE.

Figure 2: Effect of catalase and tempol, alone and in combination, on responses of juvenile and weanling arterioles to ACh and simvastatin



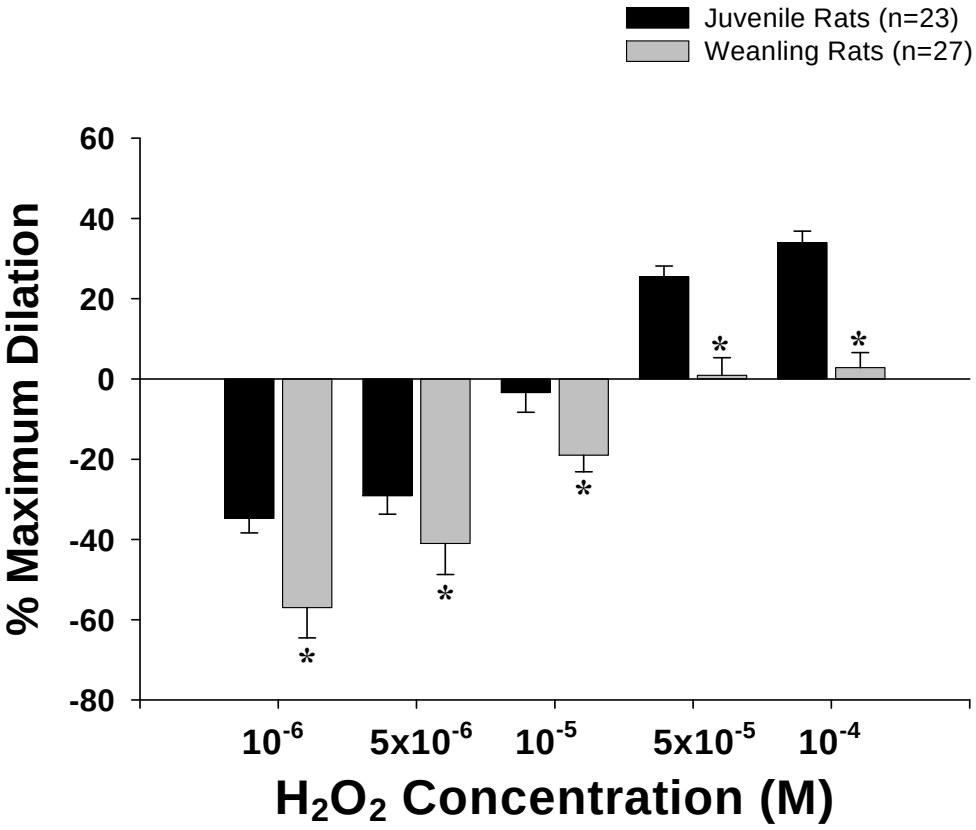
n = number of vessels. Values are given as means ± SE. * $p<0.05$ vs. Control.

Figure 3: Effect of ACh, alone or with catalase, on DCF fluorescence intensity



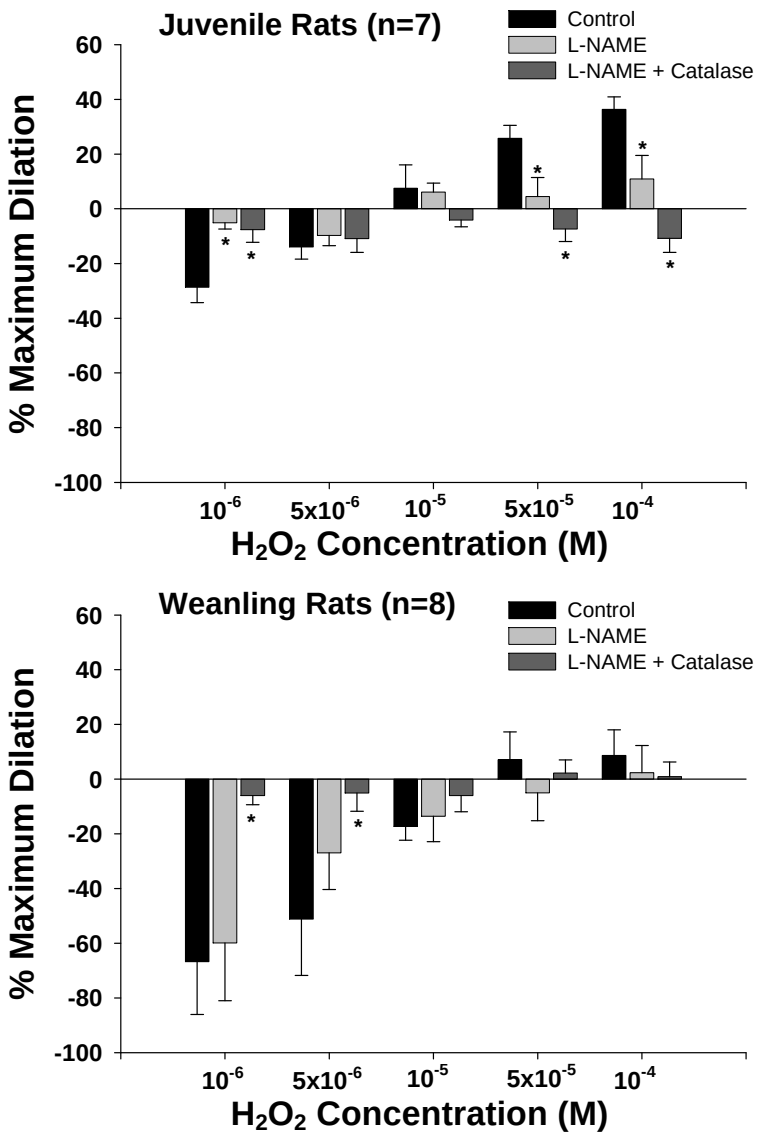
Representative microscopic images showing DCF fluorescence in arterioles exposed to DCFH-DA. Left: juvenile vessels. Right: weanling vessels. Bottom panel: Effect of 10^{-5} M ACh, alone or with catalase, on mean DCF fluorescence intensity in weanling and juvenile arterioles. * $p<0.05$ vs. Control in same age group. † $p<0.05$ vs. ACh in same age group. ‡$p<0.05$ vs. Control in juvenile arterioles.

Figure 4: Responses of juvenile and weanling arterioles to exogenous H_2O_2



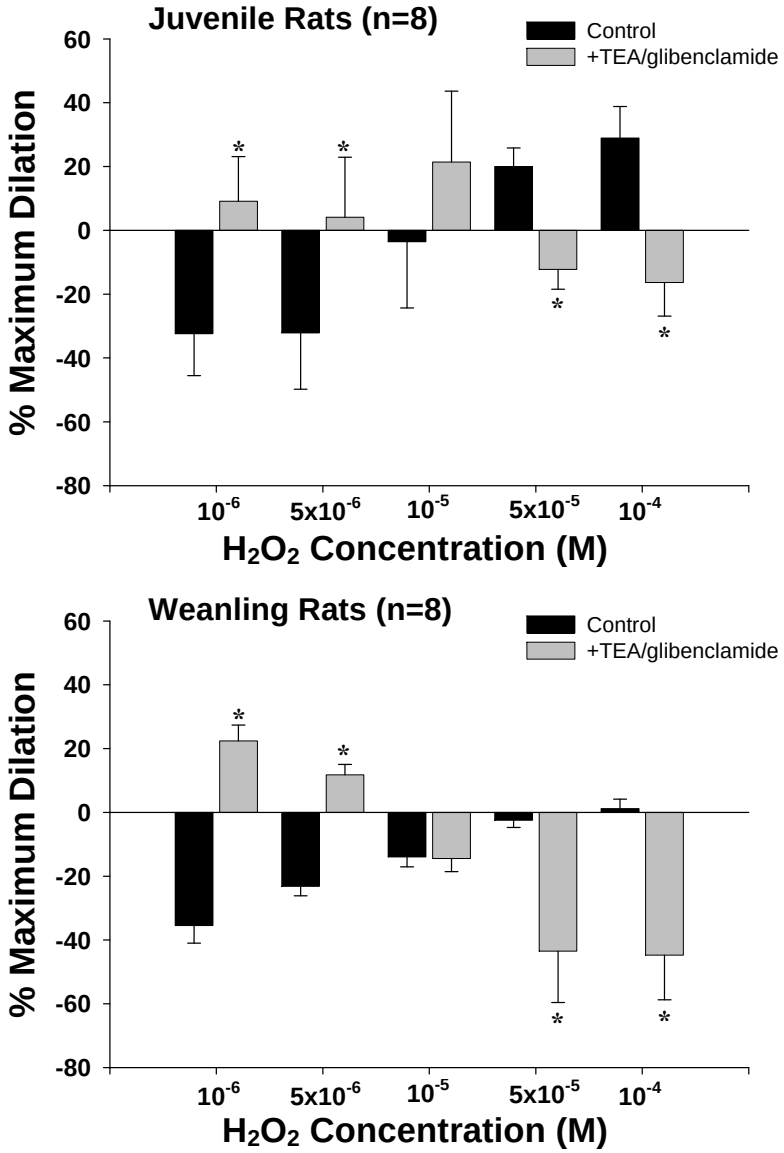
n = number of vessels. Values are given as means ± SE. * $p<0.05$ vs. juvenile arterioles.

Figure 5: Responses of juvenile and weanling arterioles to exogenous H_2O_2, before and after L-NAME treatment, alone and in combination with catalase



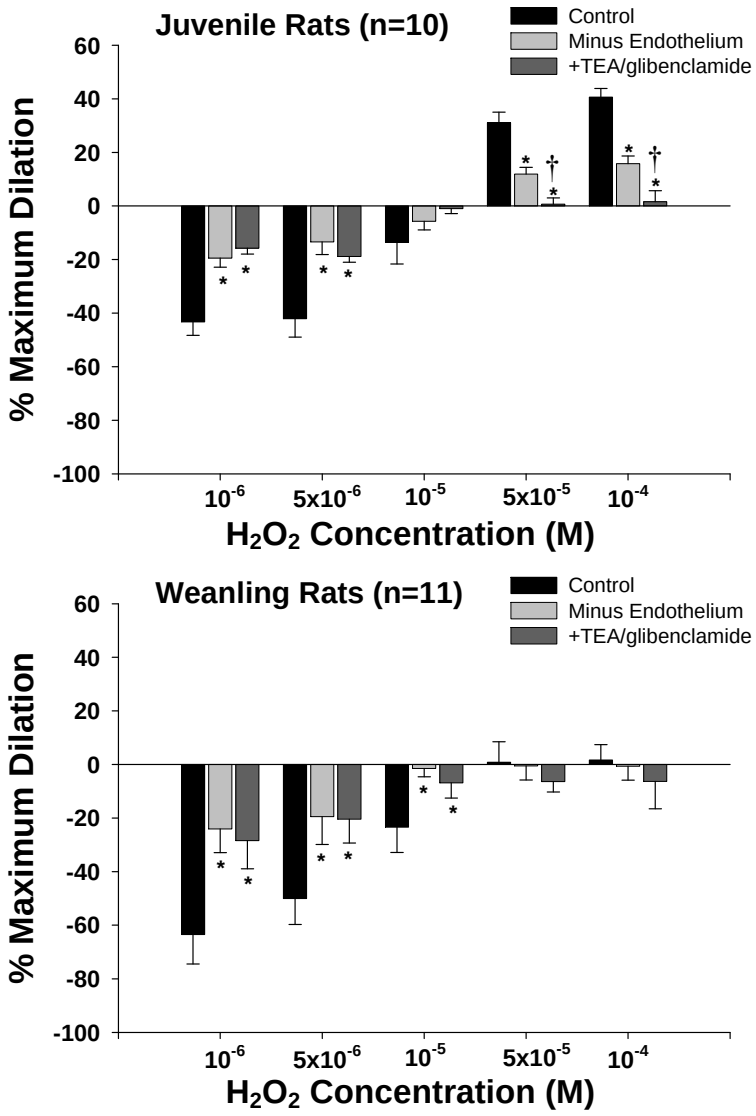
n = number of vessels. Values are given as means ± SE. * $p<0.05$ vs. Control.

Figure 6: Responses of juvenile and weanling arterioles to exogenous H_2O_2 before and after inhibition of K_{Ca} channels and K_{ATP} channels



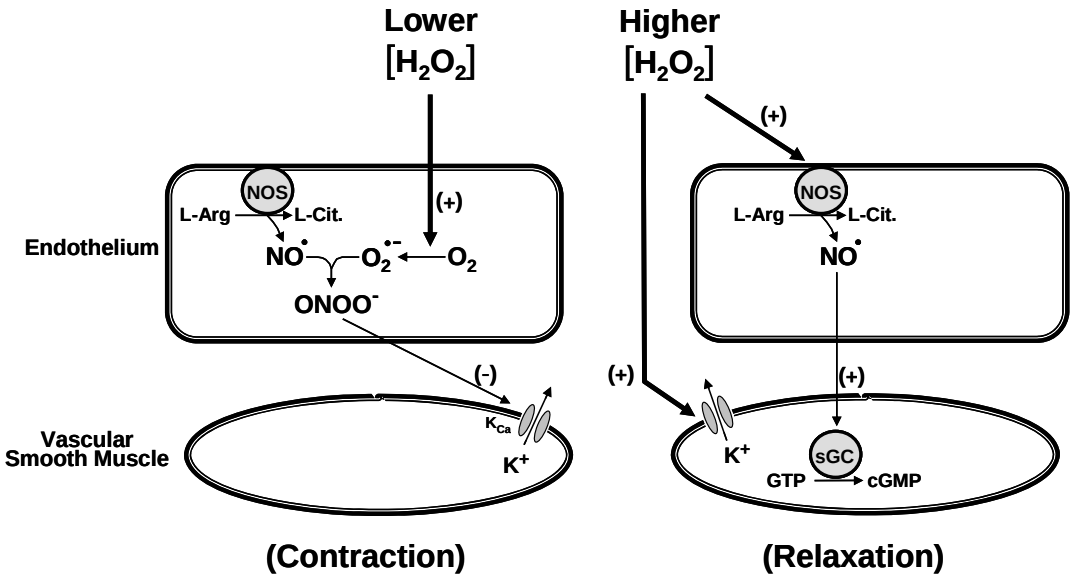
n = number of vessels. Values are given as means ± SE. * $p<0.05$ vs. Control.

Figure 7: Effect of endothelial removal on responses of juvenile and weanling arterioles to exogenous H_2O_2 before and after combined treatment with TEA and glibenclamide



n=number of vessels. Values are given as means ± SE. * $p<0.05$ vs. Control.

Figure 8: Schematic diagram illustrating possible elements of the signaling pathways through which H_2O_2 may elicit either arteriolar constriction or arteriolar dilation



NOS: Nitric oxide synthase. cGMP: Cyclic Guanosine Monophosphate. sGC: Soluble Guanylyl Cyclase. GTP: guanosine-5'-triphosphate. K_{Ca}: Ca^{2+}-activated K^+ channels. L-Arg: L-arginine. L-Cit: L-citrulline.

Discussion

Growth-related changes in microvascular endothelial function have been reported by our laboratory and others (Linderman & Boegehold, 1999; Willis & Leffler, 2001; Nurkiewicz & Boegehold, 2004; Balch Samora, 2007). In a recent study (Balch Samora, 2007), we found that skeletal muscle arterioles from mature rats exhibit endothelium-dependent dilations to ACh and simvastatin that are due in part to endothelial NO release, with vasodilator prostanoids and at least one unidentified endothelium-derived hyperpolarizing factor (EDHF) also contributing to these responses in some cases. However, in that study, we also found that the endothelium-dependent dilation of arterioles from younger (weanling) rats is not mediated by any of these factors. Since H_2O_2 can mediate endothelium-dependent dilation in the cerebral circulation of newborn pigs (Lacza *et al.*, 2002), we undertook the current study in part to determine if H_2O_2 could be playing an analogous role in the skeletal muscle circulation of weanling rats.

Contribution of H_2O_2 to endothelium-dependent responses of juvenile arterioles

Exogenous catalase greatly reduced the dilation of juvenile arterioles to both ACh and simvastatin (Figure 2). Furthermore, we found a large, catalase-sensitive component of DCF fluorescence in these vessels at rest (approximately 50% of total fluorescence) that was further increased (to approximately 63% of total fluorescence) when the vessels were challenged with ACh (Figure 3, bottom panel). These findings indicate that endogenous H_2O_2 contributes to, rather than opposes, the dilation of these vessels to ACh and simvastatin, and suggests that these agonists stimulate a relatively large increase in H_2O_2 within the arteriolar wall. Furthermore, our recent finding that endothelial removal

completely abolishes both ACh- and simvastatin-induced dilation of these arterioles (Balch Samora, 2007) indicates that this H_2O_2 is endothelial in origin.

The O_2^- from which this H_2O_2 is formed could be generated during electron transport within the mitochondria, or from the intracellular activity of various enzymes, including membrane-bound NADPH oxidase, xanthine oxidase, cyclooxygenase, cytochrome P450 monooxygenase or nitric oxide synthase (Cai & Harrison, 2000). Mitochondrial and cytosolic SODs would quickly convert this O_2^- to H_2O_2, which can diffuse across the plasma membrane and into the extracellular space (Cai & Harrison, 2000; Abid *et al.*, 2001). It is also possible that some H_2O_2 may be generated extracellularly from O_2^- formed by xanthine oxidase (Li *et al.*, 2001;Seshiah *et al.*, 2002; Coyle *et al.*, 2006). Because it is uncharged, relatively stable and freely diffusible, extracellular H_2O_2 could diffuse to nearby cells and exert its effect (Griendling & Harrison, 1999; Chen *et al.*, 2003).

Because any agonist-induced increase in H_2O_2 production must first involve increased O_2^- formation, we initially expected that in the presence of the SOD mimetic tempol, ACh or simvastatin application would trigger a greater amount of H_2O_2 production and therefore a larger dilation. However, we did not find this to be the case (Figure 2, top panel), which suggests that the intrinsic SOD capacity of these vessels may be high enough to dismutate all of the additional O_2^- that is formed in response to these agonists, such that treatment with tempol would have no additional effect.

The responses of juvenile arterioles to exogenously applied H_2O_2 were complex, with lower concentrations of H_2O_2 eliciting constriction and higher concentrations of H_2O_2 eliciting dilation (Figure 4). The constrictor responses were reduced by both NOS

inhibition (Figure 5, top panel) and endothelial removal (Figure 7, top panel), and completely abolished by inhibition of K^+ channels (Figure 6, top panel). H_2O_2 can stimulate O_2^- formation in ECs (Coyle *et al.*, 2006), and O_2^- readily interacts with endothelial NO to produce peroxynitrite ($ONOO^-$), which increases the tone of gracilis muscle arterioles by inhibition of K_{Ca} channel activity (Frisbee *et al.*, 2002). Our findings are therefore consistent with a role for $ONOO^-$ as a mediator of these constrictions. However, such a mechanism would be difficult to reconcile with our speculation that SOD activity is normally high in these vessels. Although high SOD activity could explain why tempol does not increase arteriolar responses to ACh or simvastatin, it would *reduce* the likelihood of there being excess O_2^- available to interact with NO during exposure to H_2O_2 at low concentrations. It is possible that the amount of O_2^- generated during H_2O_2 application is greater than that generated during ACh or simvastatin application, since H_2O_2 can activate NAD(P)H oxidase in both endothelium and vascular smooth muscle to produce O_2^- in a feed-forward mechanism (Li *et al.*, 2001; Seshiah *et al.*, 2002; Coyle *et al.*, 2006). In our juvenile arterioles, 10-6 M H_2O_2 may therefore increase O_2^- production to the point that it supersedes the system's capacity for dismutation of this O_2^-. With the resulting accumulation of O_2^-, there could be significant $ONOO^-$ formation if local NO levels are sufficiently high. In contrast, neither ACh nor simvastatin, at the concentrations we used, may have triggered a large enough increase in H_2O_2 to stimulate this accelerated O_2^- production.

Cseko et al (2004) found that although H_2O_2-induced constriction of gracilis muscle arterioles could be completely abolished by the prostaglandin H_2 /thromboxane A_2 (PGH_2/TxA_2) receptor antagonist SQ-29548, this H_2O_2-induced constriction was only

reduced (not abolished) by endothelial removal. This suggests that H_2O_2 increases arteriolar tone via the release of constrictor prostanoids from both the endothelium and vascular smooth muscle. The possibility that $ONOO^-$ may mediate juvenile arteriolar constriction to 10^{-6} M H_2O_2 in our current study does not necessarily conflict with these earlier findings. Cseko and colleagues did not study the effect of SQ-29548 on responses to H_2O_2 at concentrations less than 3×10^{-5} M, which is 300 times the concentration at which we suggest that H_2O_2 may lead to $ONOO^-$ formation. At 5×10^{-6} M H_2O_2, which is closer to the minimum concentration used by Cseko et al., and at still higher concentrations that actually overlap those used by Cseko et al., we found no evidence that would suggest a role for $ONOO^-$ in the constriction, raising the possibility that PGH_2/TxA_2 could be mediating the constriction at these concentrations of H_2O_2 in our study as well.

The dilation of juvenile arterioles to higher concentrations of H_2O_2 was also reduced following endothelial removal (Figure 7), implying an important role for endothelium-derived mediators in these responses as well. Endothelial removal has also been shown to reduce H_2O_2-induced dilation in mouse coronary arterioles (Thengchaisri & Kuo, 2003). However, these findings contrast with a report by Miura et al. that endothelial denudation does not affect H_2O_2-induced dilation of human coronary arterioles (Miura *et al.*, 2003). Although this may reflect species-specific differences, it could also be due to different study conditions. The coronary arterioles studied by Miura et al. were obtained from patients with various diseases (e.g., coronary artery disease, diabetes mellitus, hypertension), and therefore could have displayed some degree of EC dysfunction, including impairment of NO bioavailability, oxidative stress, and even EC

injury and apoptosis (Simionescu, 2007). It is possible that the mechanism by which H_2O_2 influences vascular tone may be altered under pathologic conditions.

As shown in the top panel of Figure 5, inhibition of NOS in intact juvenile arterioles reduced the magnitude of H_2O_2-induced dilation to a level similar to that achieved by de-endothelialization, which implicates endothelium-derived NO as a major contributor to this response. We also found that blockade of K_{Ca} and K_{ATP} channels completely abolished the residual endothelium-*independent* portion of this dilation (Figure 7, top panel), suggesting that there is also a direct relaxing effect of H_2O_2 on vascular smooth muscle in these vessels due to K^+ channel activation. This is consistent with previous studies demonstrating that H_2O_2 can induce smooth muscle hyperpolarization and vasodilation by opening K^+ channels, although the main contributing channel type (i.e., K_{Ca} vs. K_{ATP} channels) can vary with the species or vascular bed studied (Campbell *et al.*, 1996; Filipovic & Reeves, 1997; Miura *et al.*, 1999; Iida& Katusic, 2000; Matoba *et al.*, 2000).

Figure 8 summarizes the signaling events that our current findings suggest could be involved in the H_2O_2-induced constriction or dilation of rat gracilis muscle arterioles. Further studies will be necessary to critically test each of these hypothesized pathways, and to identify other cellular and biochemical events that contribute to these responses.

Absence of H_2O_2 in weanling arterioles

In contrast to our findings in juvenile arterioles, catalase had no effect on the responses of weanling arterioles to ACh or simvastatin (Figure 2, bottom panel), or on DCF fluorescence in these vessels either at rest or during ACh exposure (Figure 3,

bottom panel). Therefore, H_2O_2 apparently does not contribute to the endothelium-dependent dilation of weanling arterioles to these agonists. In further support of this conclusion, exogenous H_2O_2 did not induce dilation of weanling arterioles, even at concentrations that are clearly above maximum endogenous H_2O_2 levels (Figure 4) (Cosentino *et al.*, 1998).

L-NAME had no effect on H_2O_2-induced constriction of weanling arterioles (Figure 5, bottom panel), which is not surprising in light of earlier findings that these vessels may produce little or no NO (2). Therefore, the mechanism underlying this constriction must be different from that in juvenile arterioles (see above). In the presence of K^+ channel inhibitors, weanling arterioles became responsive to higher concentrations of H_2O_2, showing pronounced constriction (Figure 6, bottom panel). This suggests that a constrictor mechanism not involving K^+ channel inhibition is usually masked by an offsetting K^+ channel-dependent hyperpolarization of vascular smooth muscle. Curiously, blocking the two main types of K^+ channels in the weanling arterioles converted the constrictions to lower concentrations of H_2O_2 into dilations. The mechanism of this effect is not clear and will require further study.

Overall, our findings suggest that an intact endothelium is required for the full expression of both constrictor and dilator responses to H_2O_2 in vessels from both age groups. In contrast to our findings in intact weanling arterioles, application of K^+ channel blockers to endothelium-denuded weanling arterioles caused no further reduction in responses to H_2O_2 (Figure 7), implying that endothelium-derived K^+ channels are predominantly involved in mediating H_2O_2 responses in the younger animals.

In conclusion, H_2O_2 apparently contributes to the endothelium-dependent dilation of skeletal muscle arterioles from juvenile rats, but not those from weanling rats. A sizable fraction of the response to H_2O_2 appears to be endothelium-dependent in both weanling and juvenile arterioles, but smooth-muscle derived K^+ channels also contribute to this response in juvenile vessels. Whereas NO plays an important role in contributing to the juvenile arteriolar response to H_2O_2, it is not involved in weanling arteriolar response to H_2O_2.

References

Abid MR, Tsai JC, Spokes KC, Deshpande SS, Irani K, & Aird WC. (2001). Vascular endothelial growth factor induces manganese-superoxide dismutase expression in endothelial cells by a Rac1-regulated NADPH oxidase-dependent mechanism. *Faseb J* 15: 2548-2550.

Balch Samora J, Frisbee JC, & Boegehold MA. (2007). Growth-dependent changes in endothelial factors regulating arteriolar tone. *Am J Physiol Heart Circ Physiol* 292: H207-H214.

Cai H. (2005). NAD(P)H oxidase-dependent self-propagation of hydrogen peroxide and vascular disease. *Circ Res* 96: 818-822.

Cai H & Harrison DG. (2000). Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res* 87: 840-844.

Campbell WB, Gebremedhin D, Pratt PF, & Harder DR. (1996). Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ Res* 78: 415-423.

Chen K, Thomas SR, & Keaney JF, Jr. (2003). Beyond LDL oxidation: ROS in vascular signal transduction. *Free Radic Biol Med* 35: 117-132.

Coleman HA, Tare M, & Parkington HC. (2001). EDHF is not K^+ but may be due to spread of current from the endothelium in guinea pig arterioles. *Am J Physiol Heart Circ Physiol* 280: H2478-2483.

Cosentino F, Patton S, d'Uscio LV, Werner ER, Werner-Felmayer G, Moreau P, Malinski T, & Luscher TF. (1998). Tetrahydrobiopterin alters superoxide and nitric oxide release in prehypertensive rats. *J Clin Invest* 101: 1530-1537.

Coyle CH, Martinez LJ, Coleman MC, Spitz DR, Weintraub NL, & Kader KN. (2006). Mechanisms of H_2O_2-induced oxidative stress in endothelial cells. *Free Radic Biol Med* 40: 2206-2213.

Cseko C, Bagi Z, & Koller A. (2004). Biphasic effect of hydrogen peroxide on skeletal muscle arteriolar tone via activation of endothelial and smooth muscle signaling pathways. *J Appl Physiol* 97: 1130-1137.

DeLano FA, Schmid-Schonbein GW, Skalak TC, & Zweifach BW. (1991). Penetration of the systemic blood pressure into the microvasculature of rat skeletal muscle. *Microvasc Res* 41: 92-110.

Fallet RW, Bast JP, Fujiwara K, Ishii N, Sansom SC, & Carmines PK. (2001). Influence of Ca^{2+}-activated K^+ channels on rat renal arteriolar responses to depolarizing agonists. *Am J Physiol Renal Physiol* 280: F583-591.

Filipovic DM & Reeves WB. (1997). Hydrogen peroxide activates glibenclamide-sensitive K^+ channels in LLC-PK1 cells. *Am J Physiol* 272: C737-743.

Fredricks KT, Liu Y, & Lombard JH. (1994). Response of extraparenchymal resistance arteries of rat skeletal muscle to reduced PO_2. *Am J Physiol* 267: H706-715.

Fredricks KT, Liu Y, Rusch NJ, & Lombard JH. (1994). Role of endothelium and arterial K^+ channels in mediating hypoxic dilation of middle cerebral arteries. *Am J Physiol* 267: H580-586, 1994.

Frisbee JC, Maier KG, & Stepp DW. (2002). Oxidant stress-induced increase in myogenic activation of skeletal muscle resistance arteries in obese Zucker rats. *Am J Physiol Heart Circ Physiol* 283: H2160-2168.

Frisbee JC, Roman RJ, Murali Krishna U, Falck JR, & Lombard JH. (2001). Altered mechanisms underlying hypoxic dilation of skeletal muscle resistance arteries of hypertensive versus normotensive Dahl rats. *Microcirculation* 8: 115-127.

Griendling KK & Harrison DG. (1999). Dual role of reactive oxygen species in vascular growth. *Circ Res* 85: 562-563.

Iida Y & Katusic ZS. (2000). Mechanisms of cerebral arterial relaxations to hydrogen peroxide. *Stroke* 31: 2224-2230.

Klabunde RE & Anderson DE. (2002). Role of nitric oxide and reactive oxygen species in platelet-activating factor-induced microvascular leakage. *J Vasc Res* 39: 238-245.

Koller A & Bagi Z. (2004). Nitric oxide and H_2O_2 contribute to reactive dilation of isolated coronary arterioles. *Am J Physiol Heart Circ Physiol* 287: H2461-2467.

Kunsch C & Medford RM. (1999). Oxidative stress as a regulator of gene expression in the vasculature. *Circ Res* 85: 753-766.

Lacza Z, Puskar M, Kis B, Perciaccante JV, Miller AW, & Busija DW. (2002). Hydrogen peroxide acts as an EDHF in the piglet pial vasculature in response to bradykinin. *Am J Physiol Heart Circ Physiol* 283: H406-411.

Lenda DM, Sauls BA, & Boegehold MA. (2000). Reactive oxygen species may contribute to reduced endothelium-dependent dilation in rats fed high salt. *Am J Physiol Heart Circ Physiol* 279: H7-H14.

Li WG, Miller FJ, Jr., Zhang HJ, Spitz DR, Oberley LW, & Weintraub NL. (2001). H_2O_2-induced O_2 production by a non-phagocytic NAD(P)H oxidase causes oxidant injury. *J Biol Chem* 276: 29251-29256.

Linderman JR &Boegehold MA. (1996). Arteriolar network growth in rat striated muscle during juvenile maturation. *Int J Microcirc Clin Exp* 16: 232-239.

Linderman JR & Boegehold MA. (1999). Growth-related changes in the influence of nitric oxide on arteriolar tone. *Am J Physiol* 277: H1570-1578.

Lombard JH, Liu Y, Fredricks KT, Bizub DM, Roman RJ, & Rusch NJ. (1999). Electrical and mechanical responses of rat middle cerebral arteries to reduced PO_2 and prostacyclin. *Am J Physiol* 276: H509-516.

Lum H & Roebuck KA. (2001). Oxidant stress and endothelial cell dysfunction. *Am J Physiol Cell Physiol* 280: C719-741.

Marvar PJ & Boegehold MA. (2006). Dietary salt reduces hydrogen peroxide-dependent dilation in contracting muscle. *FASEB J* 20: A268.

Matoba T, Shimokawa H, Morikawa K, Kubota H, Kunihiro I, Urakami-Harasawa L, Mukai Y, Hirakawa Y, Akaike T, & Takeshita A. (2003). Electron spin resonance detection of hydrogen peroxide as an endothelium-derived hyperpolarizing factor in porcine coronary microvessels. *Arterioscler Thromb Vasc Biol* 23: 1224-1230.

Matoba T, Shimokawa H, Nakashima M, Hirakawa Y, Mukai Y, Hirano K, Kanaide H, & Takeshita A. (2000). Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J Clin Invest* 106: 1521-1530.

Miura H, Bosnjak JJ, Ning G, Saito T, Miura M, & Gutterman DD. (2003). Role for hydrogen peroxide in flow-induced dilation of human coronary arterioles. *Circ Res* 92: e31-40.

Miura H, Liu Y, & Gutterman DD. (1999). Human coronary arteriolar dilation to bradykinin depends on membrane hyperpolarization: contribution of nitric oxide and Ca^{2+}-activated K^+ channels. *Circulation* 99: 3132-3138.

Nelson MT & Quayle JM. (1995). Physiological roles and properties of potassium channels in arterial smooth muscle. *Am J Physiol* 268: C799-822.

Nurkiewicz TR & Boegehold MA. (2004). Calcium-independent release of endothelial nitric oxide in the arteriolar network: onset during rapid juvenile growth. *Microcirculation* 11: 453-462.

Oltman CL, Kane NL, Miller FJ, Jr., Spector AA, Weintraub NL, & Dellsperger KC.

(2003). Reactive oxygen species mediate arachidonic acid-induced dilation in porcine coronary microvessels. *Am J Physiol Heart Circ Physiol* 285: H2309-2315.

Seshiah PN, Weber DS, Rocic P, Valppu L, Taniyama Y, & Griendling KK. (2002). Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. *Circ Res* 91: 406-413.

Silveira LR, Pereira-Da-Silva L, Juel C, & Hellsten Y. (2003). Formation of hydrogen peroxide and nitric oxide in rat skeletal muscle cells during contractions. *Free Radic Biol Med* 35: 455-464.

Simionescu M. (2007). Implications of early structural-functional changes in the endothelium for vascular disease. *Arterioscler Thromb Vasc Biol*: 27: 266-274.

Sobey CG, Heistad DD, & Faraci FM. (1997). Mechanisms of bradykinin-induced cerebral vasodilatation in rats. Evidence that reactive oxygen species activate K^+ channels. *Stroke* 28: 2290-2294; discussion 2295.

Standen NB, Quayle JM, Davies NW, Brayden JE, Huang Y, & Nelson MT. (1989). Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science* 245: 177-180.

Tarpey MM & Fridovich I. (2001). Methods of detection of vascular reactive species: nitric oxide, superoxide, hydrogen peroxide, and peroxynitrite. *Circ Res* 89: 224-236.

Thengchaisri N & Kuo L. (2003). Hydrogen peroxide induces endothelium-dependent and -independent coronary arteriolar dilation: role of cyclooxygenase and potassium channels. *Am J Physiol Heart Circ Physiol* 285: H2255-2263.

Thiemermann C. (2003). Membrane-permeable radical scavengers (tempol) for shock, ischemia-reperfusion injury, and inflammation. *Crit Care Med* 31: S76-84.

Uluoglu C & Zengil H. (2003). Comparison of different de-endothelization procedures in the isolated rat thoracic aorta: a short communication. *Res Commun Mol Pathol Pharmacol* 113-114: 289-297.

Ungvari Z, Csiszar A, & Koller A. (2002). Increases in endothelial Ca^{2+} activate K_{Ca} channels and elicit EDHF-type arteriolar dilation via gap junctions. *Am J Physiol Heart Circ Physiol* 282: H1760-1767.

Ungvari Z, Sun D, Huang A, Kaley G, & Koller A. (2001). Role of endothelial $[Ca^{2+}]_i$ in activation of eNOS in pressurized arterioles by agonists and wall shear stress. *Am J Physiol Heart Circ Physiol* 281: H606-612.

Welsh DG & Segal SS. (2000). Role of EDHF in conduction of vasodilation along hamster cheek pouch arterioles in vivo. *Am J Physiol Heart Circ Physiol* 278: H1832-1839.

Willis AP & Leffler CW. (2001). Endothelial NO and prostanoid involvement in newborn and juvenile pig pial arteriolar vasomotor responses. *Am J Physiol Heart Circ Physiol* 281: H2366-2377, 2001.

Yada T, Shimokawa H, Hiramatsu O, Kajita T, Shigeto F, Goto M, Ogasawara Y, & Kajiya F. (2003). Hydrogen peroxide, an endogenous endothelium-derived hyperpolarizing factor, plays an important role in coronary autoregulation in vivo. *Circulation* 107: 1040-1045.

Zhu J, Mori T, Huang T, & Lombard JH. (2004). Effect of high-salt diet on NO release and superoxide production in rat aorta. *Am J Physiol Heart Circ Physiol* 286: H575-583.

IV. STUDY 3: Growth-Dependent Changes in the Contribution of Carbon Monoxide to Arteriolar Function

Abstract

Endothelium-dependent dilation of skeletal muscle arterioles is mediated by factors that are yet to be identified in very young rats. We assessed the possible contribution of carbon monoxide (CO) to arteriolar dilation at two growth stages. Gracilis muscle arterioles were isolated from rats at ages 24-26 days (weanlings) and 46-48 days (juveniles). Exogenously applied CO (10^{-6} – 10^{-4} M) in the presence or absence of chromium mesoporphyrin (CrMP) constricted arterioles from both groups. Inhibition of nitric oxide synthase (NOS) with L-NAME or endothelial removal attenuated CO-induced constriction of juvenile arterioles, but these treatments had no effect on the weanling arterioles. Inhibition of K^+ channels with iberiotoxin + glibenclamide abolished the constriction of weanling arterioles, but had no effect on juvenile arterioles. CrMP had no effect on resting tone in either group, whereas the heme precursor δ-aminolevulinic acid (δ-ALA) constricted juvenile arterioles but had no effect on weanling arterioles. CrMP abolished endothelium-dependent dilations of juvenile arterioles to simvastatin and significantly reduced ACh- and simvastatin- induced dilations of weanling arterioles. The expression of heme oxygenase isoforms HO-1 and HO-2 was similar in weanling and juvenile arterioles. These findings suggest that relatively high concentrations of CO can constrict arterioles in both groups, with the primary mechanism being inhibition of endothelium-derived NO in juvenile arterioles but inhibition of K^+ channels in weanling

arterioles. Endogenous CO produced at lower concentrations may mediate endothelium-dependent dilation in both age groups.

Introduction

Studies from our laboratory have documented that postnatal growth of the microvasculature is accompanied by progressive changes in a number of factors that can influence arteriolar tone and blood flow (Linderman & Boegehold, 1996, 1999; Balch Samora *et al.*, 2007a; Balch Samora *et al.*, 2007b). For example, skeletal muscle arterioles from young rats exhibit fully-developed endothelium-dependent dilation, but these responses are not mediated by nitric oxide (NO), cyclooxygenase metabolites, or hydrogen peroxide (H_2O_2), as they are in arterioles from more mature rats (Balch Samora *et al.*, 2007a; Balch Samora *et al.*, 2007b). The chemical mediator(s) of arteriolar endothelium-dependent dilation in these younger animals have not yet been identified.

Carbon monoxide (CO), a signaling molecule that can be important for the regulation of vascular tone and blood flow under some conditions (Gonzales & Walker, 2002; Fiumana *et al.*, 2003; Kanu *et al.*, 2006), is produced via metabolism of heme by heme oxygenase (HO), with biliverdin and free iron also being generated in the process (Maines, 1997). Two of this enzyme's three known isoforms, HO-1 and HO-2, have been found in endothelial (Marks *et al.*, 1997; Parfenova *et al.*, 2001) and vascular smooth muscle (Christodoulides *et al.*, 1995; Yet *et al.*, 1997) cells. HO-1 is an inducible isoform; its expression can be dramatically increased by numerous stimuli including hypoxia, hypertension, endotoxic shock, and shear stress (Ewing *et al.*, 1994; Yet *et al.*, 1997). In contrast, HO-2 is constitutively expressed and therefore presumably

responsible for moment-to-moment changes in vascular CO production (Ding *et al.*, 1999).

Carbon monoxide is generally considered to be a vasodilator molecule (Johnson *et al.*, 1999); among other vessel types, CO has been found to relax rabbit and rat aorta (Lin & McGrath, 1988; Furchgott & Jothianandan, 1991), dog coronary arteries (Furchgott & Jothianandan, 1991), rabbit pulmonary arteries (Steinhorn *et al.*, 1994), rat afferent arterioles (Thorup *et al.*, 1999) and pial arterioles in newborn pigs (Leffler *et al.*, 1999). However, CO can also reduce NO synthesis by inhibiting NOS (White & Marletta, 1992; Pufahl & Marletta, 1993; Matsuoka *et al.*, 1994), and at least one study has demonstrated a constrictor role for CO in vivo (Marks *et al.*, 2003). The effect of CO on arteriolar tone in skeletal muscle is variable. Some investigators have found CO-dependent dilation of gracilis muscle arterioles (Kozma *et al.*, 1997), whereas others have found that both exogenously applied and endogenously formed CO elicits a constriction of these arterioles after pretreatment with phenylephrine (Johnson & Johnson, 2003). Such discrepancies may be due in part to differences in the concentration of CO reached at or within the vessel wall. For example, at high CO levels, NO release from rat afferent arterioles is suppressed, whereas at low CO levels, NO release from these vessels is maximal (Thorup *et al.*, 1999).

Because CO has been identified as an important vasoactive factor in the cerebral microcirculation of newborn pigs (Leffler *et al.*, 1999), we undertook the current study to determine if CO could be playing a similar role as a mediator of endothelium-dependent dilation in skeletal muscle arterioles of young rats. A second aim of this study was to

determine if there is a change in the mechanism(s) by which CO influences arteriolar tone during rapid juvenile growth.

Materials and Methods

Animals: All surgical and experimental procedures were approved by the West Virginia University Animal Care and Use Committee. Experiments were performed on isolated gracilis muscle arterioles from male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) of two age groups: 3-4 weeks ("weanlings") and 6-7 weeks ("juveniles").

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight), with supplemental anesthetic (10% of original dose) administered if needed. The right carotid artery was cannulated with polyethylene tubing for measurement of mean arterial pressure, which was assessed immediately before removal of the gracilis arteriole.

Preparation of Isolated Vessels: An arteriolar branch of the femoral artery supplying the gracilis muscle was removed, handling only the surrounding connective tissue to minimize vessel stretching or damage. The rat was sacrificed immediately after vessel removal by intracardiac injection of sodium pentobarbital. The excised vessel was placed in warmed physiological salt solution (PSS) equilibrated with 21% O_2, 5% CO_2, and 74% N_2, and having the following composition (mM): 119 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 1.6 $CaCl_2$, 1.18 NaH_2PO_4, 24 $NaHCO_3$, 0.026 EDTA, and 5.5 glucose. After isolation, each vessel was prepared for in vitro video microscopy as previously described (Fredricks *et al.*, 1994). Briefly, the vessel was mounted in a heated (37º C) chamber that

allowed for its lumen and exterior surface to be perfused and superfused, respectively, with PSS from separate reservoirs. The vessel was cannulated at both ends with glass micropipettes (50- and 70-µm tip diameters for weanling and juvenile vessels, respectively) and secured to the inflow and outflow pipettes using 9-0 nylon suture. Any side branches were ligated with a single strand teased from 6-0 silk suture. The inflow pipette was connected to a reservoir perfusion system for control of intralumenal pressure and flow. The vessel was then extended to its in situ length and equilibrated at 80% of the animal's mean arterial pressure to approximate its normal in vivo perfusion pressure (DeLano *et al.*, 1991).

Vessel diameter was measured using an onscreen video micrometer. Any vessel that did not demonstrate endothelial viability, as judged by pronounced dilation to 10^{-7} M acetylcholine (ACh, Sigma Chemical, St. Louis, MO), was not used in this study. Changes in vessel diameter to all agonists and inhibitors (see below) were made under static, zero-flow conditions after a 30-minute equilibration period with continuous perfusion. Resting vascular tone under zero-flow conditions was calculated as $(\Delta D/D_{max})$ x 100, where ΔD is the diameter increase from rest in response to Ca^{2+}-free PSS (30-40 minute equilibration with Ca^{2+}-free bath solution), and D_{max} is the maximum diameter measured under these conditions.

Agonists: Endothelium-dependent dilation was elicited by application of ACh or simvastatin (Merck Research Laboratories, Rathway, NJ) at bath concentrations of 10^{-5} or 10^{-7} M (Balch Samora *et al.*, 2007a; Balch Samora *et al.*, 2007b). Simvastatin was first activated by alkaline lysis (5.25 ml of 0.1 N NaOH per 140 mg, dissolved in 3.5 ml of ETOH) at 50ºC for 2 h. The resulting solution was then diluted to a volume of 35 ml

with PBS, and neutralized to pH 7.4 with HCl. One-ml aliquots of this solution were then serially diluted with PBS for addition to the vessel bath.

Carbon monoxide-saturated solution (C.P. Grade 99.5%, Airgas Mid America) was prepared as described by Johnson and Johnson (2003). Briefly, ice-cold distilled H_2O was vigorously bubbled with CO through a glass gas diffuser for 30 minutes to prepare a 10^{-2} M solution. Increasing volumes of this solution were incrementally added to the vessel bath to produce final CO concentrations of 10^{-6}, 10^{-5}, or 10^{-4} M. After each application, the vessel was allowed to return to its control diameter before the next CO application. Control experiments were performed using ice cold H_2O bubbled with N_2 (Airgas Mid America). To stimulate endogenous CO production through HO activity, the heme precursor δ-aminolevulinic acid (δ-ALA, Frontier Scientific, Logan, UT) was added to the bath at a concentration of 10^{-6} M (Andresen *et al.*, 2006).

Inhibitors: Some vessels were pretreated with chromium (III) mesoporphyrin IX chloride (CrMP, Frontier Scientific), a photostable competitive inhibitor of HO (Vreman *et al.*, 1993). Although there are many commercially-made metalloporphyrins that inhibit HO, CrMP was chosen because it is the most selective inhibitor of HO activity (Appleton *et al.*, 1999). A 10^{-2} M stock solution of CrMP in 0.1 N NaOH was diluted in the bath to produce a final concentration of 10^{-5} M (Andresen *et al.*, 2006). During exogenous CO application, endogenous CO production was also inhibited with CrMP to more directly assess intrinsic vascular responsiveness to CO.

To evaluate possible interactions of exogenous or endogenous CO with the NO system, the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, Sigma) was added to the bath at a concentration of 10^{-4} M (Frisbee *et al.*, 2001), alone or in the

presence of CrMP. Both CrMP and L-NAME were administered for 20 minutes before vessels were challenged with agonists.

The contribution of potassium (K^+) channels to arteriolar responses was assessed by using iberiotoxin (Ibtx, Sigma) (Nelson & Quayle, 1995), and the antidiabetic sulphonylurea, glibenclamide (Glib, Sigma) (Lombard *et al.*, 1999). We used a 10^{-7} M bath concentration of Ibtx to selectively block Ca^{2+}-activated K^+ (K_{Ca}) channels (Nelson & Quayle, 1995; Frisbee *et al.*, 2001), and 10^{-6} M glibenclamide to selectively block ATP-sensitive K^+ (K_{ATP}) channels (Standen *et al.*, 1989).

Endothelial Denudation: To determine the role of the endothelium in mediating arteriolar responses to exogenous CO, the endothelium was removed in some experiments by mechanical abrasion (Uluoglu & Zengil, 2003). The pipette tip at each end of the vessel was gently advanced and then retracted through the vessel lumen three times to ensure elimination of the endothelium. We have previously verified that this method successfully denudes the endothelium of gracilis muscle arterioles without affecting the underlying smooth muscle (Balch Samora *et al.*, 2007a). To verify that smooth muscle function was intact following denudation in the current experiments, vasoconstrictor responses to 10^{-5} M phenylephrine (Sigma) and vasodilator responses to 10^{-5} M sodium nitroprusside (SNP, Sigma) were assessed before and after the denudation procedure. Only those vessels with unchanged responses to both agonists were included in the final data set.

HO-1 and HO-2 protein measurements: Femoral and gracilis artery/arteriole segments were harvested from weanling and juvenile rats, snap frozen in liquid N_2, and stored at -80ºC until analysis. Protein expression was determined by Western blotting, as

described by Liu et al (Liu *et al.*, 2002). Briefly, tissue samples were thawed at room temperature and lysed in Price Laemmli buffer (Laemmli, 1970), boiled and then sonicated. Twenty μg of the extracted protein was separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The membranes were incubated for 1 h in PBS containing Tween 20 (0.05%) and nonfat milk (5%). Blots were incubated with either rabbit anti-HO-1 or anti-HO-2 polyclonal antibody (1:5000 dilution) for 1 h. Membranes were then incubated for 1h with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:5,000 dilution). Blots were developed by ECL and relative protein levels were quantified by scanning densitometry. β-actin levels were also assessed to verify equal protein loading among lanes.

Statistics: All data are presented as mean ± SE. For all analyses, a probability value of $p < 0.05$ was considered to be statistically significant. Dilation in response to Ca^{2+}-free PSS is expressed as percent increase from control diameter. Differences among the means of individual experimental groups were determined by ANOVA/Newman-Keuls test, or by an unpaired Student's *t*-test when two independent means were compared.

Results

General characteristics of all rats from which vessels were removed for functional studies are reported in Table 1. Age, body weight, and mean arterial pressure were significantly greater in juvenile rats than in weanling rats. Table 1 also summarizes the general characteristics of all arterioles utilized for functional studies. Resting and passive diameters of arterioles from juvenile rats were significantly greater than those of

arterioles from weanling rats. However, the level of spontaneous tone that developed with vessel pressurization was modestly but significantly greater in the weanling arterioles.

CrMP had no effect on resting arteriolar diameters for either age group (Table 2). δ-ALA significantly reduced the resting diameter of juvenile arterioles (by 25 ± 1%), but not weanling arterioles. Exogenous CO caused significant arteriolar constriction in both groups, with weanling arterioles constricting significantly more than juvenile arterioles in response to 10^{-6} M and 10^{-5} M CO (Figure 1). The vehicle alone (equilibrated with N_2) had no effect in either group (data not presented). CrMP was without effect on these responses to exogenous CO in either group, indicating that alterations in endogenous CO production were not influencing arteriolar behavior under these conditions (data not shown).

L-NAME treatment reduced the resting diameter of both juvenile arterioles (from 55 ± 5 to 40 ± 5 μm, a 27% average diameter reduction) and weanling arterioles (from 35 ± 4 to 25 ± 3 μm, a 29% average diameter reduction). In contrast, L-NAME had no effect on resting diameters in either group if CrMP was present (44 ± 6 μm before vs. 44 ± 4 μm after L-NAME in juveniles and 30 ± 3 μm before vs. 37 ± 4 μm after L-NAME in weanlings). However, L-NAME abolished the constrictor responses of juvenile arterioles to exogenous CO whether or not CrMP was present (Figure 2, top panels). L-NAME had no effect on weanling arteriole responses to CO at any concentration (Figure 3, top left panel), although it tended to reduce these responses to CO in the presence of CrMP (significantly at 10^{-6} M CO) (Figure 3, top right panel).

Treatment with Ibtx + Glib reduced the resting diameters of both juvenile arterioles (from 51 ± 6 to 35 ± 4 μm, a 31% average diameter reduction) and weanling

arterioles (from 43 ± 2 to 29 ± 3 μm, a 33% average diameter reduction). CrMP did not modify the effect of Ibtx + Glib on the tone of juvenile arterioles, but completely abolished this effect on the tone of weanling arterioles (data not shown). For juvenile arterioles, Ibtx + Glib had no effect on CO-induced constriction, whether or not CrMP was present (Figure 2, bottom panels). However, for weanling arterioles, Ibtx + Glib abolished the constrictor responses to exogenous CO at all concentrations, whether or not CrMP was present (Figure 3, bottom panels).

Endothelial removal tended to reduce the resting diameters of both juvenile arterioles (55 ± 2 μm before vs. 49 ± 2 μm after de-endothelialization) and weanling arterioles (26 ± 3 μm vs. 20 ± 2 μm), but these reductions did not reach statistical significance. Endothelial removal had no effect on CO-induced constriction of weanling arterioles, but significantly reduced these responses in juvenile arterioles (Figure 4).

Although treatment with CrMP did not affect the dilation of juvenile arterioles to ACh, it abolished the dilation of these vessels to simvastatin (Figure 5, top panel). However, CrMP treatment reduced the dilation of weanling arterioles to both ACh and simvastatin (bottom panel). Addition of L-NAME after CrMP treatment abolished dilation to ACh and had no further effect on simvastatin responses in juvenile arterioles (Figure 6, top panel), and had no further effect on weanling arteriolar responses to ACh or simvastatin (bottom panel). In juvenile arterioles, treatment with Ibtx + Glib following CrMP abolished responses to ACh and had no further effect on responses to simvastatin (Figure 7, top panel). In weanling arterioles, treatment with Ibtx + Glib following CrMP had no effect on ACh responses, but reduced the dilation to 10^{-5} M simvastatin (bottom panel).

Western blot analysis of femoral artery/ gracilis arteriole homogenates revealed no difference in either HO-1 or HO-2 protein expression between weanling and juvenile rats (Figure 8).

Table 1: General characteristics of all rats and vessels used for functional studies

Animal Characteristics	**Weanlings**	**Juveniles**
N	47	52
Age (days)	25.3 ± 0.3	43.8 ± 0.5 *
Body Weight (g)	62 ± 1	168 ± 2 *
MAP (mmHg)	76 ± 1	97 ± 2 *
Vessel Characteristics	**Weanlings**	**Juveniles**
n	47	52
Resting Diameter (µm)	34 ± 1	51 ± 2 *
Passive Diameter (µm)	57 ± 1	79 ± 2 *
Resting Vascular Tone (%)	41 ± 2	35 ± 2 *

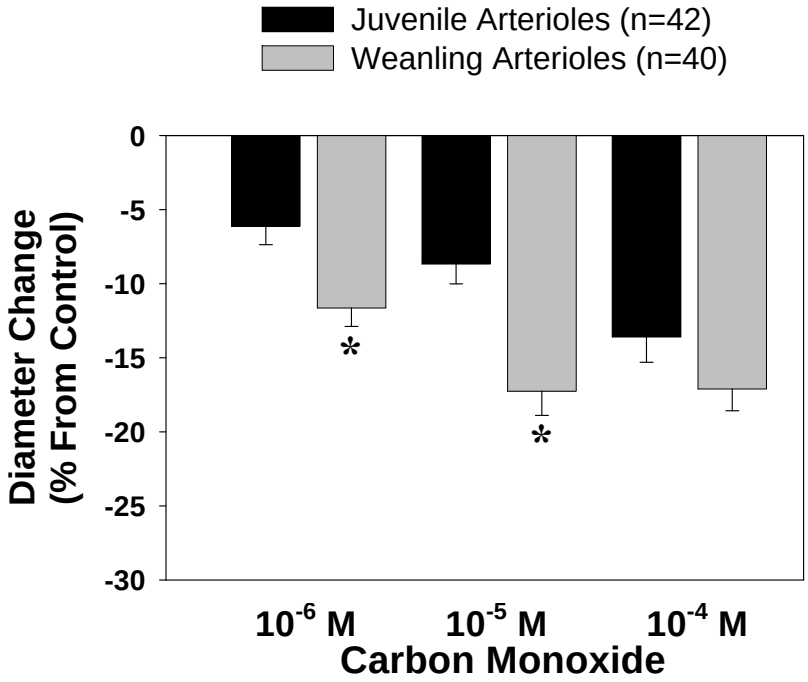
MAP = mean arterial pressure. Values are given as means ± SE. * $P<0.05$ vs. weanling group.

Table 2: Summary of resting arteriolar diameters under control conditions and after treatment with CrMP or δ-ALA

	n	Control	CrMP (10^{-5} M)	n	Control	δ-ALA (10^{-6} M)
Juveniles	16	47 ± 3	47 ± 3	10	52 ± 3	39 ± 6 *
Weanlings	17	32 ± 2	37 ± 3	7	35 ± 3	38 ± 4

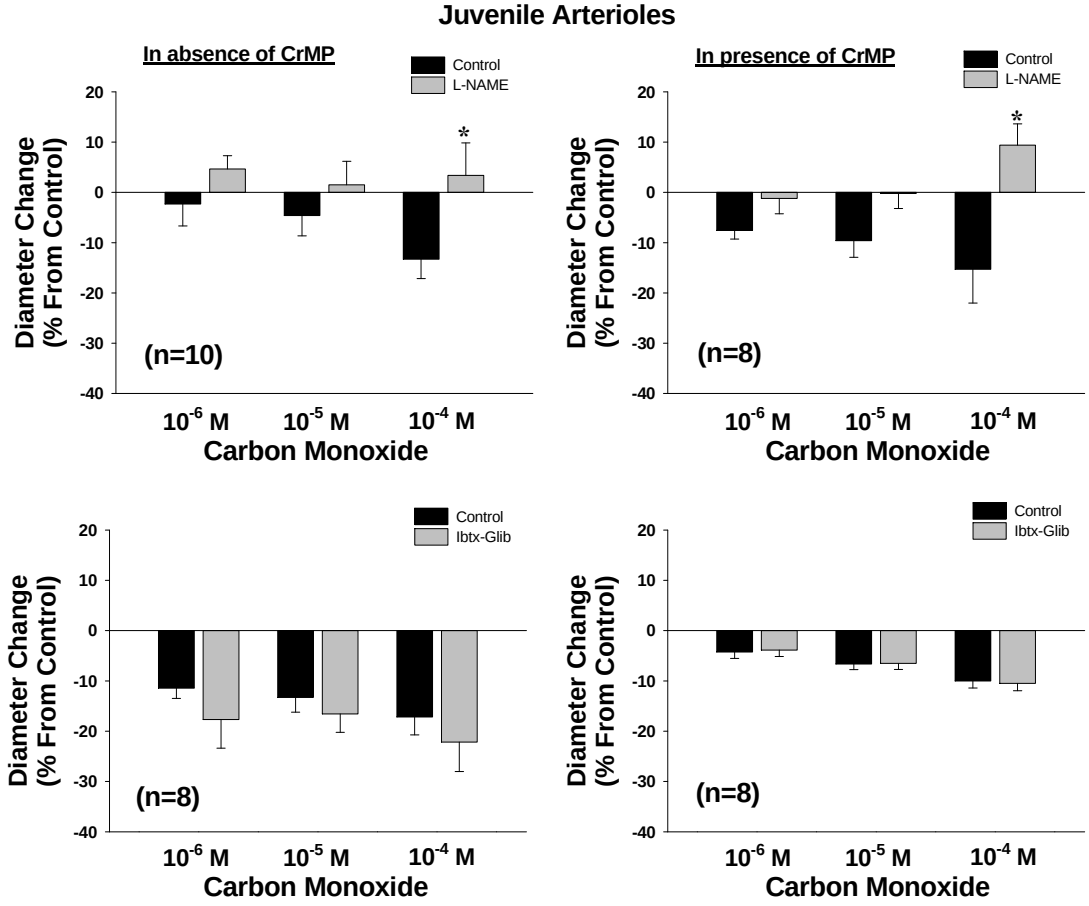
Values are given as means ± SE. * $P<0.05$ vs. Control.

Figure 1: Responses of gracilis muscle arterioles from juvenile and weanling rats to increasing concentrations of exogenous CO



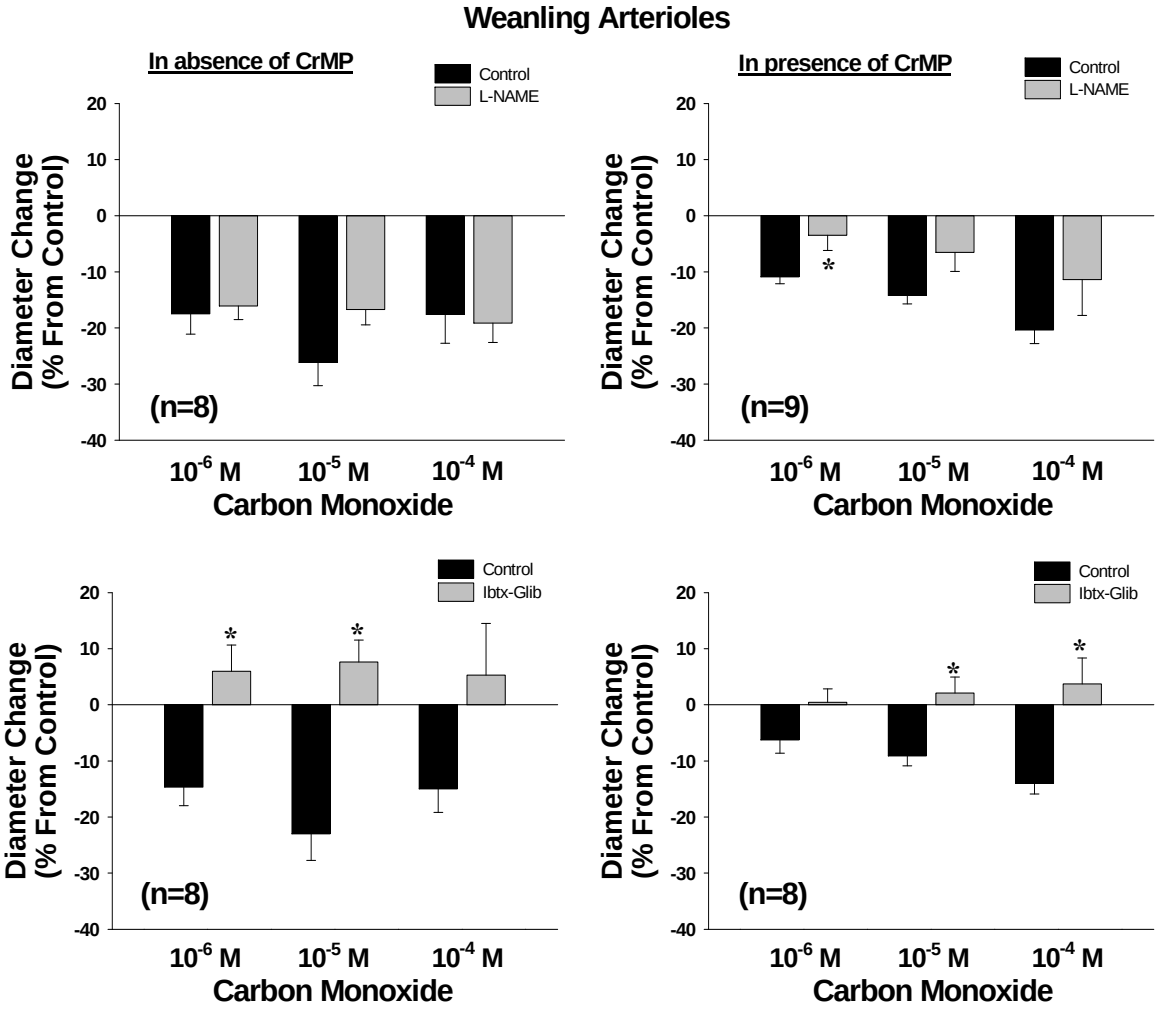
n = number of vessels. Values are given as means ± SE. * $p<0.05$ vs. Juvenile response.

Figure 2: Responses of arterioles from juvenile rats to exogenous CO alone or in the presence of CrMP, L-NAME, Ibtx/Glib or combined treatments



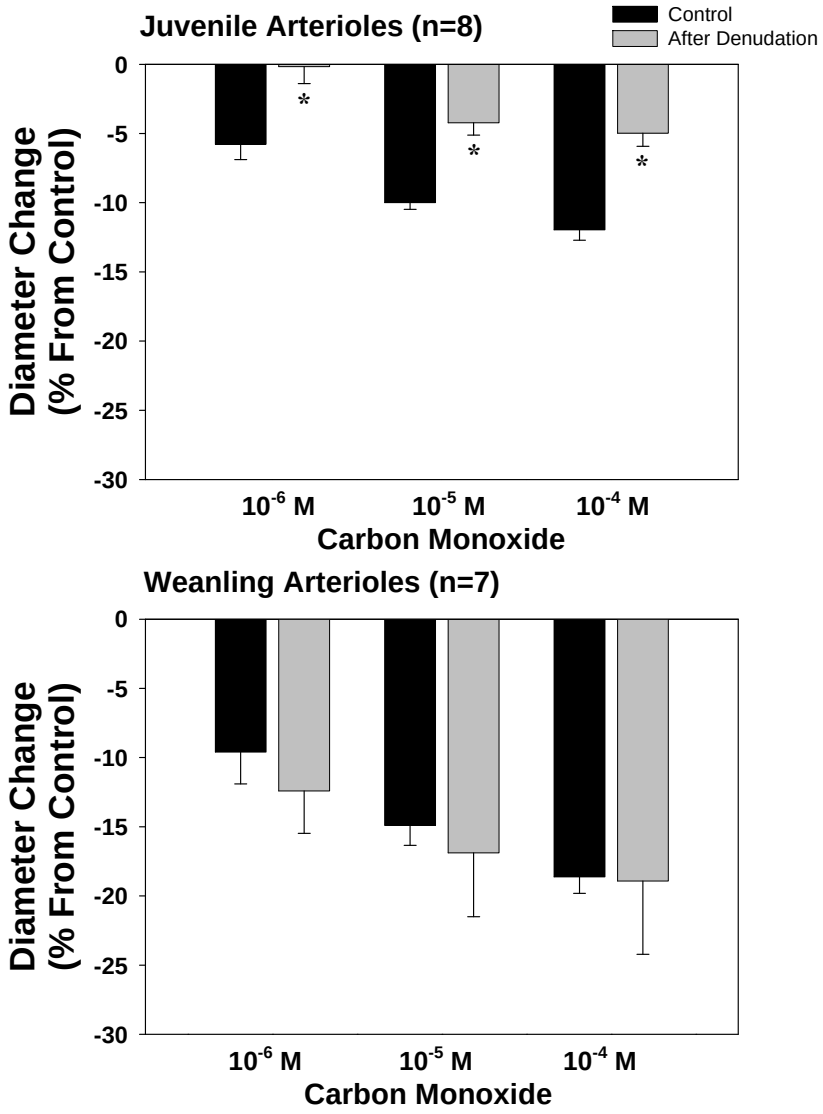
Responses of arterioles from juvenile rats to exogenous carbon monoxide before and after NOS inhibition with N^{ω}-nitro-L-arginine methyl ester (L-NAME) alone (top left panel) or in the presence of CrMP (top right panel), and before and after inhibition of K_{Ca} and K_{ATP} channels with iberiotoxin (Ibtx) + glibenclamide (Glib) alone (bottom left panel) or in the presence of CrMP (bottom right panel). n = number of vessels. Values are given as means ± SE. * $p<0.05$ vs. Control.

Figure 3: Responses of arterioles from weanling rats to exogenous CO alone or in the presence of CrMP, L-NAME, Ibtx/Glib or combined treatments



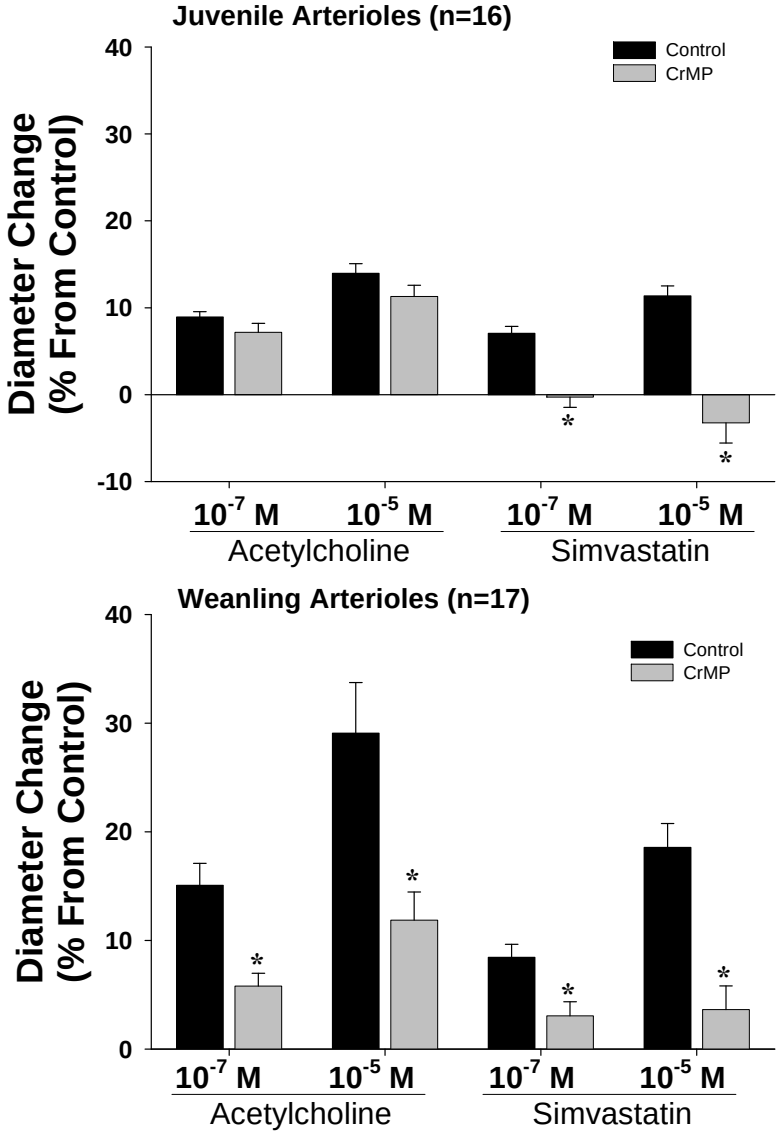
Responses of arterioles from weanling rats to exogenous carbon monoxide before and after NOS inhibition with N^{ω}-nitro-L-arginine methyl ester (L-NAME) alone (top left panel) or in the presence of CrMP (top right panel), and before and after inhibition of K_{Ca} and K_{ATP} channels with iberiotoxin (Ibtx) + glibenclamide (Glib) alone (bottom left panel) or in the presence of CrMP (bottom right panel). n = number of vessels. Values are given as means ± SE. * $p<0.05$ vs. Control.

Figure 4: Effect of endothelial removal on responses of juvenile and weanling arterioles to exogenous CO



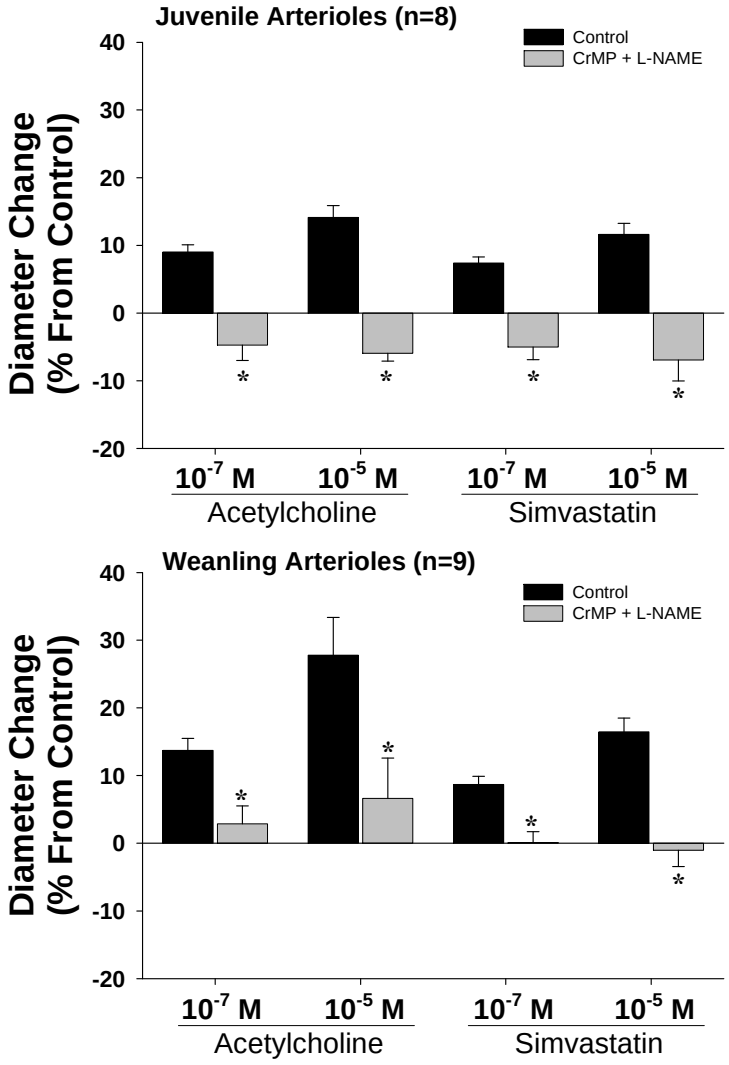
n=number of vessels. Values are given as means ± SE. * $p<0.05$ vs. Control.

Figure 5: Effect of CrMP on responses of juvenile and weanling arterioles to ACh and simvastatin



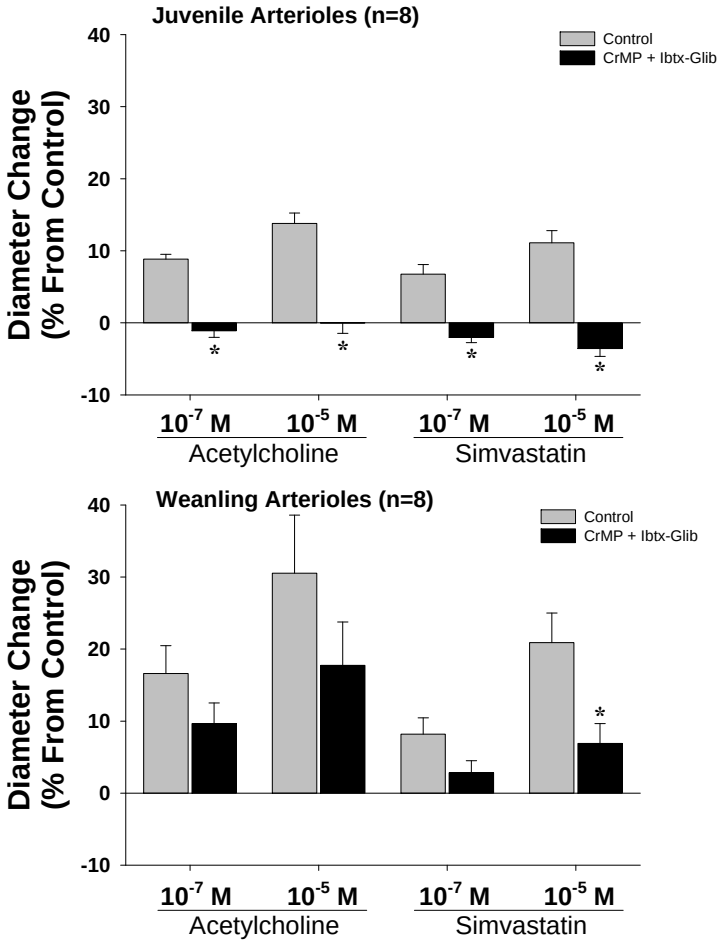
n = number of vessels. Values are given as means ± SE. * $p<0.05$ vs. Control.

Figure 6: Responses of arterioles from juvenile and weanling rats to ACh and simvastatin before and after treatment with CrMP and L-NAME



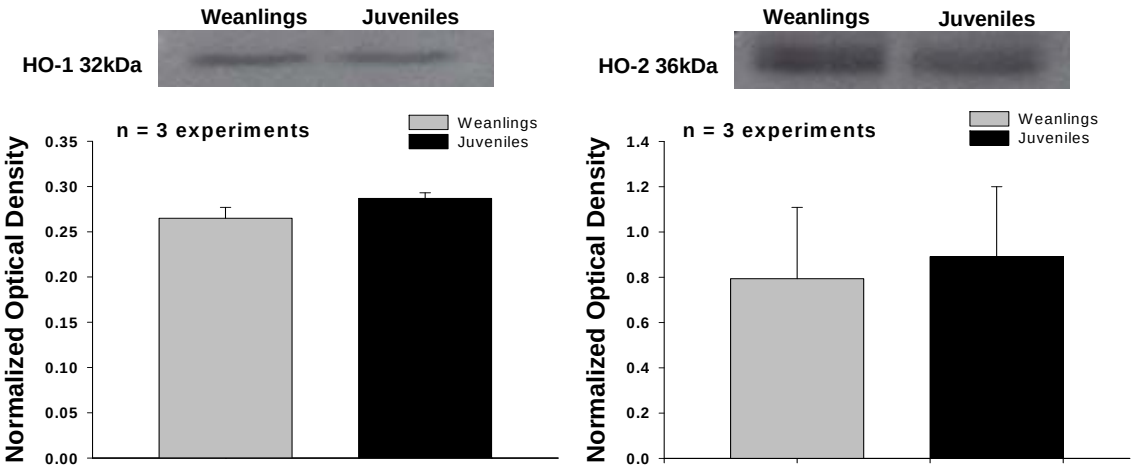
n = number of vessels. Values are given as means ± SE. * $p<0.05$ vs. Control.

Figure 7: Responses of arterioles from juvenile and weanling rats to ACh and simvastatin before and after treatment with CrMP and Ibtx-Glib



n = number of vessels. Values are given as means ± SE. * $p<0.05$ vs. Control.

Figure 8: Assessment of heme oxygenase (HO)-1 and -2 protein expression by Western blot in weanling and juvenile femoral artery/ gracilis arteriole segments



Discussion

Growth-related changes in microvascular endothelial function have been reported by our laboratory and others (Nelson & Quayle, 1995; Linderman & Boegehold, 1999; Willis & Leffler, 2001; Balch Samora *et al.*, 2007a). In our recent investigations on gracilis muscle arterioles (Balch Samora *et al.,* 2007a, 2007b), we ruled out NO, prostanoids, cytochrome P-450 metabolites and H_2O_2 as possible mediators of endothelium-dependent dilation in weanling rats. Since CO has been found to mediate endothelium-dependent dilation of cerebral arterioles in newborn pigs (Leffler *et al.*, 1999), we undertook the current study in part to determine if CO could be playing an analogous role in the skeletal muscle circulation of weanling rats.

Juvenile Response

The HO inhibitor CrMP had no effect on the resting diameters of juvenile arterioles (Table 2), suggesting that under the conditions of our study, basal CO release in these vessels is insufficient to influence resting smooth muscle tone. This is similar to previous findings in rat tail artery (Wang *et al.*, 1997), aorta (Kozma *et al.*, 1999) and femoral artery (Kozma *et al.*, 1999), whereas treatment with CrMP has been found to dilate newborn pig pial arterioles (Leffler *et al.*, 1999). Still others have demonstrated that CrMP promotes constriction of gracilis muscle arterioles in the presence of L-NAME (Kozma *et al.*, 1997; Kozma *et al.*, 1999). These variable effects may reflect differences among vascular beds or species, but may also be related to some nonspecific effects of metalloporphyrin HO inhibitors, such as NOS inhibition or activation (Luo & Vincent, 1994; Chakder *et al.*, 1996; Grundemar & Ny, 1997; Appleton *et al.*, 1999).

Treatment with L-NAME reduced the diameter of juvenile arterioles under normal resting conditions, but not after CrMP treatment. This suggests that some level of HO activity must be present for there to be an influence of basally-released NO on resting tone. This confirms findings in other vessel types (Foresti *et al.*, 2002), but the exact mechanism of this synergistic effect is not known. CO is not a required cofactor or co-substrate for NOS, but the smooth muscle signaling pathways through which CO and NO act appear to share many common elements, including cGMP activation (Foresti *et al.*, 2002). It is possible that basally-released CO, even at concentrations too low to exert a direct effect on resting vascular tone (see above), could act to "prime" the NO signaling pathway in vascular smooth muscle, thereby increasing smooth muscle sensitivity to basally-released NO. Further studies are required to critically test this hypothesis.

The heme precursor δ-ALA increases arteriolar CO production in a dose-dependent manner (Leffler *et al.*, 2003) and our finding that 1μM δ-ALA reduces juvenile arteriole diameters by ~25% (Table 2) demonstrates that this increase in endogenous CO production triggers an increase in vascular tone. δ-ALA-induced constriction was previously reported in a study on much larger arterioles from rat gracilis muscle (Johnson & Johnson, 2003), although that effect was more modest (a constriction of ~7% in response to 80μM δ-ALA). The difference in responsiveness to δ-ALA between the two studies may reflect a difference in HO activity or substrate availability between different branch orders within the arteriolar network. In the current study, exogenous CO at concentrations from 10^{-6} to 10^{-4} M also caused arteriolar constriction (Figure 1), which is consistent with the earlier study on larger gracilis muscle arterioles (Johnson & Johnson, 2003).

We observed constriction of gracilis muscle arterioles taken from juvenile rats in response to exogenous CO, ranging from 10^{-6} - 10^{-4} M (Figure 1). In phenylephrine-pretreated rat gracilis arterioles, exogenous CO in the range of 10^{-6} - 10^{-3} M also produces vasoconstriction (Johnson & Johnson, 2003), but in an initial rapid communication, Kozma et al (1997) reported that exogenous CO elicits the dilation of gracilis muscle arterioles pretreated with L-NAME in the presence and absence of CrMP. Two years later, this same group reported that exogenous CO (10^{-4} M) does not produce dilation of L-NAME pretreated arterioles not previously exposed to CrMP (Kozma *et al.*, 1999). The reasons for these conflicting findings are not readily apparent. In our juvenile arterioles, exogenous CO promotes vasoconstriction that is sensitive to L-NAME at the highest CO concentration (Figure 2), which agrees with findings by Johnson and Johnson (2003). There is mounting evidence that relatively high concentrations of CO induce constriction by inhibiting NO release (White & Marletta, 1992; Puhfal & Marletta, 1993; Thorup *et al.*, 1999; Foresti *et al.*, 2002; Johnson & Johnson, 200). Our finding that CO-induced constriction in juvenile arterioles is abolished by L-NAME (Figure 2, top panels) and dramatically reduced after our endothelial denudation procedure (Figure 4, top panel) is consistent with this mechanism. A comparison of the two data sets reveals that endothelial denudation was actually less effective than L-NAME in reducing arteriolar responses to 10^{-5} M and 10^{-4} M CO. This unexpected finding may indicate that some of the NO inhibited by CO is of smooth muscle origin, or it may simply be due to incomplete removal of the endothelium. However, the latter possibility appears less

likely given that this denudation procedure completely abolished responses to ACh in these vessels.

Simultaneous blockade of K_{Ca} and K_{ATP} channels had no effect on CO-induced constriction of juvenile arterioles (Figure 2, bottom panels), indicating that a change in K^+ channel activity normally does not mediate or attenuate CO-induced constriction in juvenile arterioles.

Whereas CrMP had no effect on the ACh-induced dilation of these vessels, it completely abolished dilator responses to simvastatin (Figure 5, top panel). This result is somewhat intriguing as it suggests that endogenous CO may be involved in this endothelium-dependent *dilation*, whereas, as described above, increasing HO substrate availability (with δ-ALA) or exogenously applying CO produced arteriolar *constriction*. Such divergent results may be due to differences in the actual periarteriolar CO achieved by these experimental procedures. In isolated rat renal afferent arterioles, CO at lower concentrations (0.01-0.1 μM) triggers maximal NO release, whereas CO at higher concentrations suppresses NO release (Thorup *et al.*, 1999). In the rat gracilis muscle arterioles studied here, perhaps simvastatin induces significantly less CO production than δ-ALA treatment, and leads to arteriolar wall CO levels that are less than that achieved with exogenous CO in the range of 10^{-6} - 10^{-4} M. If this is correct, than the constriction of these vessels to exogenous CO in the range of 10^{-6} - 10^{-4} M (Figure 1) may reflect a relatively large increase in arteriolar wall CO levels, similar to or greater than that triggered by δ-ALA. Furthermore, because CrMP completely abolishes the dilation to simvastatin (Figure 5, top), CrMP may have had some non-specific effects in this study, as we previously demonstrated that part of the simvastatin response in these vessels is

due to endothelial NO release (Balch Samora *et al.*, 2007a). In fact, some of the demonstrated effects of metalloporphyrin HO inhibitors include either NOS inhibition (Luo & Vincent, 1994) or activation (Chakder *et al.*, 1996), or inhibition of sGC (Grundemar & Ny, 1997).

Combined treatment with CrMP + L-NAME abolished the dilation of juvenile arterioles to ACh as well as simvastatin (Figure 6, top panel). We previously demonstrated that these responses are entirely endothelium-dependent, and that L-NAME treatment alone reduces juvenile arteriole responses to ACh and simvastatin by ~90% and ~60%, respectively (Balch Samora *et al.*, 2007a). Our current findings, taken in context of these previous findings, suggest that although simvastatin releases endothelium-derived CO in addition to NO, ACh does not.

Combined K^+ channel inhibition abolished ACh-induced dilations in the presence of CrMP (Figure 7, top panel). Because CrMP alone abolished simvastatin dilations (Figure 5), combined K^+ inhibition after CrMP treatment had no further effect. We previously demonstrated that inhibition of both K_{ATP} and K_{Ca} channels significantly reduced responses to ACh (by 60% - 70%) and completely abolished responses to simvastatin in juvenile arterioles (Balch Samora *et al.*, 2007a). Combined treatment with CrMP abolished the residual dilation to ACh, which is surprising since CrMP treatment alone had no effect on ACh-induced dilation. This finding may be attributable to some nonspecific effect of CrMP in these experiments.

Weanling Response

As with juvenile arterioles, CrMP treatment had no effect on the resting tone of weanling arterioles (Table 2), suggesting that CO does not influence basal tone in the arterioles from younger rats. However, unlike juvenile arterioles, the tone of weanling arterioles was unaffected by δ-ALA. This suggests that even under conditions that lead to increased substrate for or activation of HO, CO is not produced in a sufficient amount to alter the basal tone of arterioles at this earlier stage of growth.

Leffler and colleagues (1999) have reported that exogenous CO dilates cerebral arterioles of newborn pigs in vivo. In contrast, we found that exogenous CO constricts the weanling arterioles studied here (Figure 1). Although this could reflect normal differences between species or vascular beds, it is also possible that, as discussed above, differences in CO concentration reached within the arteriolar wall could explain these divergent observations. Whereas the CO-induced dilations in newborn pigs occurred at superfusate concentrations ranging from 10^{-11} to 10^{-7} M, with maximal dilation at 10^{-9} M (Leffler *et al.*, 1999), we observed constrictions to CO at bath concentrations between 10^{-6} and 10^{-4} M. Although there is limited information regarding CO levels in vivo, tissue contents of CO are reported to be 1-50 pmol/mg fresh weight (Vreman *et al.*, 2000), which others have calculated to be approximately $1 \times 10^{-6} – 5 \times 10^{-5}$ M (Johnson & Johnson, 2003). As discussed above, CO can either dilate or constrict arterioles, depending on its effective concentration (Thorup *et al.*, 1999).

Although weanling arterioles did not dilate in response to exogenous CO, we found a reduced dilator response to ACh and simvastatin in the presence of CrMP (Figure 5, bottom panel), suggesting that a portion of this endothelium-dependent dilation is due

to endogenous CO release. In these experiments, endogenously produced CO within the arteriolar wall may have been within the range of concentrations reached by Leffler et al., which could produce dilation, rather than the higher concentrations we applied exogenously (10^{-6} to 10^{-4} M), which resulted in constriction.

In contrast to juvenile arterioles, L-NAME did not affect CO-induced constriction of weanling arterioles (Figure 3, top panels), suggesting that a decrease in NO production does not mediate these responses. Although L-NAME did reduce the resting diameter of the weanling arterioles, this did not happen after CrMP treatment, which was similar to our findings in juvenile arterioles and suggests that some level of HO activity is also required for basal NO to modulate resting arteriolar tone at this earlier stage of growth.

In contrast to our findings in the juvenile arterioles, combined inhibition of K^+ channels with iberiotoxin and glibenclamide abolished CO-induced constriction of weanling arterioles, both alone and in the presence of CrMP (Figure 3, bottom panels). This suggests that CO constricts weanling arterioles through K^+ channel inhibition and depolarization of the vascular smooth muscle (VSM). Endothelial denudation did not affect CO-induced constriction of these vessels (Figure 4, bottom panel), which is also consistent with inhibition of smooth muscle K^+ channel activity being the predominant mechanism of CO-induced arteriolar constriction at this age.

HO-1 and HO-2 have previously been identified in both the vascular smooth muscle and endothelium of gracilis muscle arterioles (Johnson *et al.*, 2002). However, we found no differences between weanling and juvenile rats in femoral artery/gracilis arteriole HO-1 or HO-2 protein levels (Figure 8). This suggests that changes in the regulation of HO activity, rather than its expression, underlie the differences we found

between weanling and juvenile arterioles. Leffler's group found that regulation of CO production in the pig cerebral circulation can involve changes in both heme availability and HO-2 catalytic activity (Leffler *et al.*, 2003).

Conclusion

In summary, the role of CO in regulating arteriolar tone appears to change during postnatal growth. Exogenously applied CO in a range of 10^{-6} – 10^{-4} M results in arteriolar constriction mediated by K^+ channel inhibition and vascular smooth muscle depolarization in weanling vessels, but by NO inhibition in juvenile vessels. Endogenously produced CO mediates the endothelium-dependent dilation of arterioles from both age groups, but this role for CO may be more widespread in weanling arterioles. Such differences underscore the importance of further studies to define the effect of maturation on microvascular function to more fully understand how tissue demands can be met throughout postnatal growth.

References

Andresen JJ, Shafi NI, Durante W & Bryan RM, Jr. (2006). Effects of carbon monoxide and heme oxygenase inhibitors in cerebral vessels of rats and mice. *Am J Physiol Heart Circ Physiol* 291, H223-230.

Appleton SD, Chretien ML, McLaughlin BE, Vreman HJ, Stevenson DK, Brien JF, Nakatsu K, Maurice DH & Marks GS. (1999). Selective inhibition of heme oxygenase, without inhibition of nitric oxide synthase or soluble guanylyl cyclase, by metalloporphyrins at low concentrations. *Drug metabolism and disposition: the biological fate of chemicals* 27, 1214-1219.

Balch Samora J, Frisbee J & Boegehold M. (2007a). Growth-dependent changes in endothelial factors regulating arteriolar tone. *American journal of physiology* 292, H207-H214.

Balch Samora J, Frisbee JC & Boegehold MA. (2007b). Hydrogen peroxide emerges as a regulator of arteriolar tone during microvascular network growth. *Faseb J* 21, A 494.

Chakder S, Rathi S, Ma XL & Rattan S. (1996). Heme oxygenase inhibitor zinc protoporphyrin IX causes an activation of nitric oxide synthase in the rabbit internal anal sphincter. *The Journal of pharmacology and experimental therapeutics* 277, 1376-1382.

Christodoulides N, Durante W, Kroll MH & Schafer AI. (1995). Vascular smooth muscle cell heme oxygenases generate guanylyl cyclase-stimulatory carbon monoxide. *Circulation* 91, 2306-2309.

DeLano FA, Schmid-Schonbein GW, Skalak TC & Zweifach BW. (1991). Penetration of the systemic blood pressure into the microvasculature of rat skeletal muscle. *Microvascular research* 41, 92-110.

Ding Y, McCoubrey WK, Jr. & Maines MD. (1999). Interaction of heme oxygenase-2 with nitric oxide donors. Is the oxygenase an intracellular 'sink' for NO? *European journal of biochemistry / FEBS* 264, 854-861.

Ewing JF, Raju VS & Maines MD. (1994). Induction of heart heme oxygenase-1 (HSP32) by hyperthermia: possible role in stress-mediated elevation of cyclic 3':5'-guanosine monophosphate. *The Journal of pharmacology and experimental therapeutics* 271, 408-414.

Fiumana E, Parfenova H, Jaggar JH & Leffler CW. (2003). Carbon monoxide mediates vasodilator effects of glutamate in isolated pressurized cerebral arterioles of newborn pigs. *Am J Physiol Heart Circ Physiol* 284, H1073-1079.

Foresti R, Green CJ, Motterlini R. Nitric oxide and the heme oxygenase/carbon monoxide system: cooperation in the control of vascular function. In: *Carbon Monoxide and Cardiovascular Functions*. Boca Raton, FL: CRC Press, 2002, p. 111-124.

Fredricks KT, Liu Y & Lombard JH. (1994). Response of extraparenchymal resistance arteries of rat skeletal muscle to reduced PO2. *The American journal of physiology* 267, H706-715.

Frisbee JC, Roman RJ, Murali Krishna U, Falck JR & Lombard JH. (2001). Altered mechanisms underlying hypoxic dilation of skeletal muscle resistance arteries of hypertensive versus normotensive Dahl rats. *Microcirculation* 8, 115-127.

Furchgott RF & Jothianandan D. (1991). Endothelium-dependent and -independent vasodilation involving cyclic GMP: relaxation induced by nitric oxide, carbon monoxide and light. *Blood vessels* 28, 52-61.

Gonzales RJ & Walker BR. (2002). Role of CO in attenuated vasoconstrictor reactivity of mesenteric resistance arteries after chronic hypoxia. *Am J Physiol Heart Circ Physiol* 282, H30-37.

Grundemar L & Ny L. (1997). Pitfalls using metalloporphyrins in carbon monoxide research. *Trends in pharmacological sciences* 18, 193-195.

Johnson FK & Johnson RA. (2003). Carbon monoxide promotes endothelium-dependent constriction of isolated gracilis muscle arterioles. *American journal of physiology* 285, R536-541.

Johnson FK, Teran FJ, Prieto-Carrasquero M & Johnson RA. (2002). Vascular effects of a heme oxygenase inhibitor are enhanced in the absence of nitric oxide. *Am J Hypertens* 15, 1074-1080.

Johnson RA, Kozma F & Colombari E. (1999). Carbon monoxide: from toxin to endogenous modulator of cardiovascular functions. *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica [et al* 32, 1-14.

Kanu A, Whitfield J & Leffler CW. (2006). Carbon monoxide contributes to hypotension-induced cerebrovascular vasodilation in piglets. *Am J Physiol Heart Circ Physiol* 291, H2409-2414.

Kozma F, Johnson RA & Nasjletti A. (1997). Role of carbon monoxide in heme-induced vasodilation. *European journal of pharmacology* 323, R1-2.

Kozma F, Johnson RA, Zhang F, Yu C, Tong X & Nasjletti A. (1999). Contribution of endogenous carbon monoxide to regulation of diameter in resistance vessels. *The American journal of physiology* 276, R1087-1094.

Laemmli UK. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Leffler CW, Balabanova L, Sullivan CD, Wang X, Fedinec AL & Parfenova H. (2003). Regulation of CO production in cerebral microvessels of newborn pigs. *Am J Physiol Heart Circ Physiol* 285, H292-297.

Leffler CW, Nasjletti A, Yu C, Johnson RA, Fedinec AL & Walker N. (1999). Carbon monoxide and cerebral microvascular tone in newborn pigs. *Am J Physiol* 276, H1641-1646.

Lin H & McGrath JJ. (1988). Vasodilating effects of carbon monoxide. *Drug and chemical toxicology* 11, 371-385.

Linderman JR & Boegehold MA. (1996). Arteriolar network growth in rat striated muscle during juvenile maturation. *International journal of microcirculation, clinical and experimental / sponsored by the European Society for Microcirculation* 16, 232-239.

Linderman JR & Boegehold MA. (1999). Growth-related changes in the influence of nitric oxide on arteriolar tone. *The American journal of physiology* 277, H1570-1578.

Liu XM, Chapman GB, Wang H & Durante W. (2002). Adenovirus-mediated heme oxygenase-1 gene expression stimulates apoptosis in vascular smooth muscle cells. *Circulation* 105, 79-84.

Lombard JH, Liu Y, Fredricks KT, Bizub DM, Roman RJ & Rusch NJ. (1999). Electrical and mechanical responses of rat middle cerebral arteries to reduced PO_2 and prostacyclin. *The American journal of physiology* 276, H509-516.

Luo D & Vincent SR. (1994). Metalloporphyrins inhibit nitric oxide-dependent cGMP formation in vivo. *European journal of pharmacology* 267, 263-267.

Maines MD. (1997). The heme oxygenase system: a regulator of second messenger gases. *Annu Rev Pharmacol Toxicol* 37, 517-554.

Marks GS, Brien JF & Nakatsu K. (2003). What role does the heme-- heme oxygenase--carbon monoxide system play in vasoregulation? *American journal of physiology* 285, R522-523.

Marks GS, McLaughlin BE, Vreman HJ, Stevenson DK, Nakatsu K, Brien JF & Pang SC. (1997). Heme oxygenase activity and immunohistochemical localization in bovine pulmonary artery and vein. *Journal of cardiovascular pharmacology* 30, 1-6.

Matsuoka A, Stuehr DJ, Olson JS, Clark P & Ikeda-Saito M. (1994). L-arginine and calmodulin regulation of the heme iron reactivity in neuronal nitric oxide synthase. *The Journal of biological chemistry* 269, 20335-20339.

Nelson MT & Quayle JM. (1995). Physiological roles and properties of potassium channels in arterial smooth muscle. *The American journal of physiology* 268, C799-822.

Parfenova H, Neff RA, 3rd, Alonso JS, Shlopov BV, Jamal CN, Sarkisova SA & Leffler CW. (2001). Cerebral vascular endothelial heme oxygenase: expression, localization, and activation by glutamate. *Am J Physiol Cell Physiol* 281, C1954-1963.

Pufahl RA & Marletta MA. (1993). Oxidation of N^G-hydroxy-L-arginine by nitric oxide synthase: evidence for the involvement of the heme in catalysis. *Biochemical and biophysical research communications* 193, 963-970.

Standen NB, Quayle JM, Davies NW, Brayden JE, Huang Y & Nelson MT. (1989). Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science* 245, 177-180.

Steinhorn RH, Morin FC, 3rd & Russell JA. (1994). The adventitia may be a barrier specific to nitric oxide in rabbit pulmonary artery. *The Journal of clinical investigation* 94, 1883-1888.

Thorup C, Jones CL, Gross SS, Moore LC & Goligorsky MS. (1999). Carbon monoxide induces vasodilation and nitric oxide release but suppresses endothelial NOS. *Am J Physiol* 277, F882-889.

Uluoglu C & Zengil H. (2003). Comparison of different de-endothelization procedures in the isolated rat thoracic aorta: a short communication. *Research communications in molecular pathology and pharmacology* 113-114, 289-297.

Vreman HJ, Ekstrand BC & Stevenson DK. (1993). Selection of metalloporphyrin heme oxygenase inhibitors based on potency and photoreactivity. *Pediatric research* 33, 195-200.

Vreman HJ, Wong RJ & Stevenson DK. (2000). Carbon monoxide in breath, blood, and other tissues. In *Carbon Monoxide Toxicity*, ed. Penney DG, pp. 19-50. CRC, Boca Raton, FL.

Wang R, Wang Z & Wu L. (1997). Carbon monoxide-induced vasorelaxation and the underlying mechanisms. *British journal of pharmacology* 121, 927-934.

White KA & Marletta MA. (1992). Nitric oxide synthase is a cytochrome P-450 type hemoprotein. *Biochemistry* 31, 6627-6631.

Willis AP & Leffler CW. (2001). Endothelial NO and prostanoid involvement in newborn and juvenile pig pial arteriolar vasomotor responses. *American journal of physiology* 281, H2366-2377.

Willis D, Tomlinson A, Frederick R, Paul-Clark MJ & Willoughby DA. (1995). Modulation of heme oxygenase activity in rat brain and spleen by inhibitors and donors of nitric oxide. *Biochemical and biophysical research communications* 214, 1152-1156.

Yet SF, Pellacani A, Patterson C, Tan L, Folta SC, Foster L, Lee WS, Hsieh CM & Perrella MA. (1997). Induction of heme oxygenase-1 expression in vascular smooth muscle cells. A link to endotoxic shock. *The Journal of biological chemistry* 272, 4295-4301.

V. STUDY 4: Increased Myogenic Responsiveness of Skeletal Muscle Arterioles with Juvenile Growth

Abstract

Previous studies from this laboratory suggest that during maturation, rapid microvascular growth is accompanied by changes in some of the mechanisms responsible for regulation of tissue blood flow. To further define these changes, we studied isolated gracilis muscle arterioles from Sprague Dawley rats at approximately 27 days of age ("weanlings") and approximately 42 days of age ("juveniles") to test the hypothesis that myogenic mechanisms for the control of arteriolar tone are altered with growth. When studied at their respective *in vivo* pressures, the myogenic index (instantaneous slope of the active pressure-diameter curve) for juvenile arterioles was greater than that for weanling arterioles. Endothelial denudation, or PGH_2/TXA_2 receptor antagonism (SQ-29548) without denudation, significantly reduced the myogenic responsiveness of juvenile arterioles over a wide range of pressures, but had no consistent effects on the myogenic responsiveness of weanling arterioles. Inhibition of heme oxygenase with chromium (III) mesoporphyrin IX chloride had no effect on the myogenic activity of arterioles from either group. These findings suggest that arteriolar growth during juvenile maturation is accompanied by an increase in myogenic responsiveness, possibly because endothelium-derived PGH_2 or TXA_2 assumes a role in reinforcing myogenic activity over this period. The results of this study further support the idea that alterations in the mechanisms of vascular tone regulation occur with growth and maturation, and that

these changes may have significant implications for the regulation of tissue perfusion during microvascular development.

Introduction

Postnatal growth of the microvasculature in skeletal muscle is accompanied by progressive changes in vessel structure, in lumenal pressure, flow and shear stress, and in other factors that can influence either resting arteriolar tone or the capacity for acute adjustments in this tone (Aquin *et al.*, 1980; Sarelius *et al.*, 1981; Zweifach *et al.*, 1981; Wang & Prewitt, 1991; Linderman & Boegehold, 1996, 1998, 1999). This is not surprising, since tissue growth during this period is associated with changing metabolic demands that should require corresponding changes in the gain of local blood flow control mechanisms (Hudlicka, 1985; Kelly *et al.*, 1991; Krijgsveld *et al.*, 2001).

The myogenic behavior of resistance vessels contributes importantly to local blood flow regulation (Davis, 1993; Davis & Hill, 1999). In most vascular beds, this myogenic activity is the direct result of stretch-dependent changes in smooth muscle contractility, although endothelium-derived factors can modulate the magnitude of this activity (Harder, 1987; Katusic *et al.*, 1987; Rubanyi, 1988; Kuo *et al.*, 1991). We have consistently found that the specific factors that mediate endothelium-dependent control of arteriolar tone can change during juvenile growth (Linderman & Boegehold, 1998, 1999; Nurkiewicz & Boegehold, 2004; Balch Samora *et al.*, 2007a; Balch Samora *et al.*, 2007b), but the impact of such changes on arteriolar myogenic behavior has not been investigated. In addition, there may be changes in the intrinsic responsiveness of vascular smooth muscle to stretch during rapid network growth. In a previous study, we

found that when pressurized to their respective *in vivo* pressures, isolated gracilis muscle arterioles from weanling rats develop a significantly higher level of spontaneous tone than arterioles from juvenile rats (Balch Samora *et al.*, 2007a), suggesting that arteriolar smooth muscle responsiveness to myogenic stimuli could be greater in the younger rats. We undertook the current study to more directly investigate the possibility that alterations in myogenic responsiveness, or in the ability to modulate this responsiveness, may occur during microvascular network growth in skeletal muscle arterioles.

Materials and Methods

Animals: All surgical and experimental procedures were approved by the West Virginia University Animal Care and Use Committee. Experiments were performed on isolated gracilis muscle arterioles from male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) of two age groups: 3-4 weeks ("weanlings") and 6-7 weeks ("juveniles").

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight), with supplemental anesthetic (10% of original dose) administered if needed. The right carotid artery was cannulated with polyethylene tubing for measurement of mean arterial pressure, which was assessed immediately before removal of the gracilis supply arteriole.

Preparation of Isolated Vessels: An arteriolar branch of the femoral artery supplying the gracilis muscle was removed, handling only the surrounding connective tissue to minimize vessel stretching or damage. The rat was then sacrificed by intracardiac injection of sodium pentobarbital. The vessel was placed in warmed physiological salt solution (PSS) equilibrated with 21% O_2, 5% CO_2, and 74% N_2 and

having the following composition (mM): 119 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 1.6 $CaCl_2$, 1.18 NaH_2PO_4, 24 $NaHCO_3$, 0.026 EDTA, and 5.5 glucose. After isolation, each vessel was prepared for *in vitro* video microscopy as previously described (Fredricks *et al.*, 1994). Briefly, the vessel was mounted in a heated (37º C) chamber that allowed its lumen and exterior surface to be perfused and superfused, respectively, with PSS from separate reservoirs. The vessel was cannulated at both ends with glass micropipettes (50- and 70-µm tip diameters for weanling and juvenile vessels, respectively) and secured to the inflow and outflow pipettes using 9-0 nylon suture. Any side branches were ligated with a single strand teased from 6-0 silk suture. The inflow pipette was connected to a reservoir perfusion system for control of intralumenal pressure and flow. The vessel was then extended to its *in situ* length and equilibrated at 80% of the animal's mean arterial pressure to approximate its *in vivo* perfusion pressure (DeLano *et al.*, 1991).

Experimental Protocols: Vessel diameter was measured using an onscreen video micrometer. During equilibration, all vessels developed spontaneous tone. Any vessel that did not demonstrate endothelial viability, as judged by pronounced dilation to 10^{-7} M acetylcholine (ACh, Sigma Chemical, St. Louis, MO), was not used in the study. Changes in vessel diameter to all agonists and inhibitors were made under static, zero-flow conditions after a 30-minute equilibration period with continuous perfusion.

To evaluate the myogenic responsiveness of each arteriole, the perfusate outflow line from the vessel was clamped and the height of perfusion reservoir was increased or decreased to vary intralumenal pressure, in 20-mmHg steps, between 40 and 140 mmHg. Steady-state vessel diameter was measured before and then 3-5 minutes after each pressure change. The order in which the different pressure steps were applied was as follows: 80→100→120→140→60→40 mmHg. After the active arteriolar diameter

responses to each pressure change were measured, the superfusate was replaced with Ca^{2+}-free PSS, and, after full vessel relaxation, passive diameter was determined at each of the pressure steps previously used.

Endothelial Denudation: To determine the role of the vascular endothelium in modulating myogenic activity, the endothelium was removed in some experiments by mechanical abrasion (Uluoglu & Zengil, 2003). To accomplish this, the pipette tip at each end of the vessel was gently advanced and then withdrawn through the vessel lumen at least three times to ensure elimination of the endothelium. After vessel responses to each step change in intralumenal pressure were determined under control conditions, the endothelium was removed and responses to each pressure step were re-evaluated. We have previously verified that this method successfully denudes the endothelium of gracilis muscle arterioles without affecting the underlying smooth muscle (Balch Samora *et al.*, 2007a). To verify that smooth muscle function was intact following denudation in the current experiments, vasoconstrictor responses to 10^{-5} M phenylephrine (Sigma) and vasodilator responses to 10^{-5} M sodium nitroprusside (SNP, Sigma) were assessed before and after the denudation procedure. Only those vessels with unchanged responses to both agonists were included in the final data set.

Inhibitors and Agonists: All chemicals were purchased from Sigma unless otherwise specified. Because previous investigations have documented a role for constrictor prostanoids in modulating the myogenic activity of skeletal muscle arterioles (Hill *et al.*, 1990; Huang *et al.*, 1993; Huang & Koller, 1997; Ungvari & Koller, 2000), pressure-diameter relationships in some vessels were investigated under control conditions and then in the presence of the prostaglandin H_2/thromboxane A_2 (PGH_2/TxA_2) receptor antagonist SQ-29548 (10^{-6} M) (Cseko *et al.*, 2004). Since

endogenously produced carbon monoxide (CO) can also modulate the myogenic activity in isolated gracilis arterioles (Zhang *et al.*, 2001), pressure-diameter relationships in other vessels were assessed before and after exposure to the selective heme oxygenase (HO) inhibitor chromium (III) mesoporphyrin IX chloride (CrMP, Frontier Scientific). For these experiments, a 10^{-2} M stock solution of CrMP in 0.1 N NaOH was diluted in the bath to produce a final concentration of 10^{-5} M (Andresen *et al.*, 2006). Vessels were incubated with either SQ-29548 or CrMP for 20 minutes before reassessing pressure-diameter curves.

Data and Statistical Analysis: The myogenic index (MI), which is an indicator of the relative slope of the active pressure-diameter relationship for an arteriole at a given pressure, was determined using the following equation: $MI = 100 \bullet [(r_f - r_i)/r_i]/(P_f - P_i)$, where r_f is the final radius, r_i is the initial radius, P_f is the final intralumenal pressure and P_i is initial intralumenal pressure. The more powerful the myogenic responsiveness, the more negative the MI.

All data are presented as mean ± SE. For all analyses, a probability value of $p < 0.05$ was considered to be statistically significant. Dilation in response to Ca^{2+}-free PSS is expressed as percent increase from control diameter. Differences between the means of individual experimental groups were determined by ANOVA/Newman-Keuls test, or by an unpaired Student's *t*-test when two means were compared.

Results

General characteristics of all rats from which vessels were removed and studied are reported in Table 1. Age, body weight, and mean arterial pressure were significantly greater in juvenile rats than in weanling rats. Table 1 also summarizes the general

characteristics of all vessels studied, measured at equilibration pressures of 61 ± 1 mmHg for weanling arterioles and 77 ± 2 mmHg for juvenile arterioles (80% of mean arterial pressure, which approximates steady-state *in vivo* pressures for these vessels). Resting and passive diameters of arterioles from juvenile rats were significantly greater than those of arterioles from weanling rats, but at these "*in vivo"* equilibration pressures, resting vascular tone was significantly less in the juvenile arterioles than weanling arterioles.

Arterioles from both age groups exhibited clear myogenic activity, as judged by a constriction to increases in intralumenal pressure above 80 mmHg in juvenile arterioles and maintenance of a constant diameter at intralumenal pressures above 80 mmHg in weanling arterioles (Figure 1). Calculated myogenic indices were similar for weanling and juvenile arterioles at pressures between 40 and 80 mmHg, but significantly more negative for juvenile arterioles at pressures of 100 and 120 mmHg, indicating a greater myogenic responsiveness (Figure 2, top panel). When intralumenal pressures were normalized to each age group's estimated *in vivo* arteriolar pressure, the difference in myogenic responsiveness between weanling and juvenile arterioles was even more profound, especially at pressures closest to their respective *in vivo* values (Figure 2, bottom panel).

As shown in Figure 3 (left panels), endothelial denudation significantly reduced the steady-state diameter of juvenile arterioles at pressures of 60 and 80 mmHg, whereas denudation shifted the myogenic indices of these vessels to less negative values (indicating reduced myogenic responsiveness) between 80 and 120 mmHg. In contrast, endothelial denudation only had a significant effect on the steady-state diameter of weanling arterioles at 140 mmHg (a slight increase), and had no consistent effect on the myogenic index of these vessels (Figure 3, right panels).

Whereas the PGH_2/TXA_2 receptor antagonist SQ-29548 did not change the steady-state diameter of juvenile arterioles at any pressure, it had an effect on the myogenic index of these vessels that was similar to that of endothelial removal: a shift to less negative values at pressures between 80 and 120 mmHg (Figure 4, left panels). In contrast, SQ-29548 tended to increase the steady state diameter of weanling arterioles (a significant effect at 40, 60 and 140 mmHg), but had no significant effect on the myogenic index for these vessels at any pressure (Figure 4, right panels).

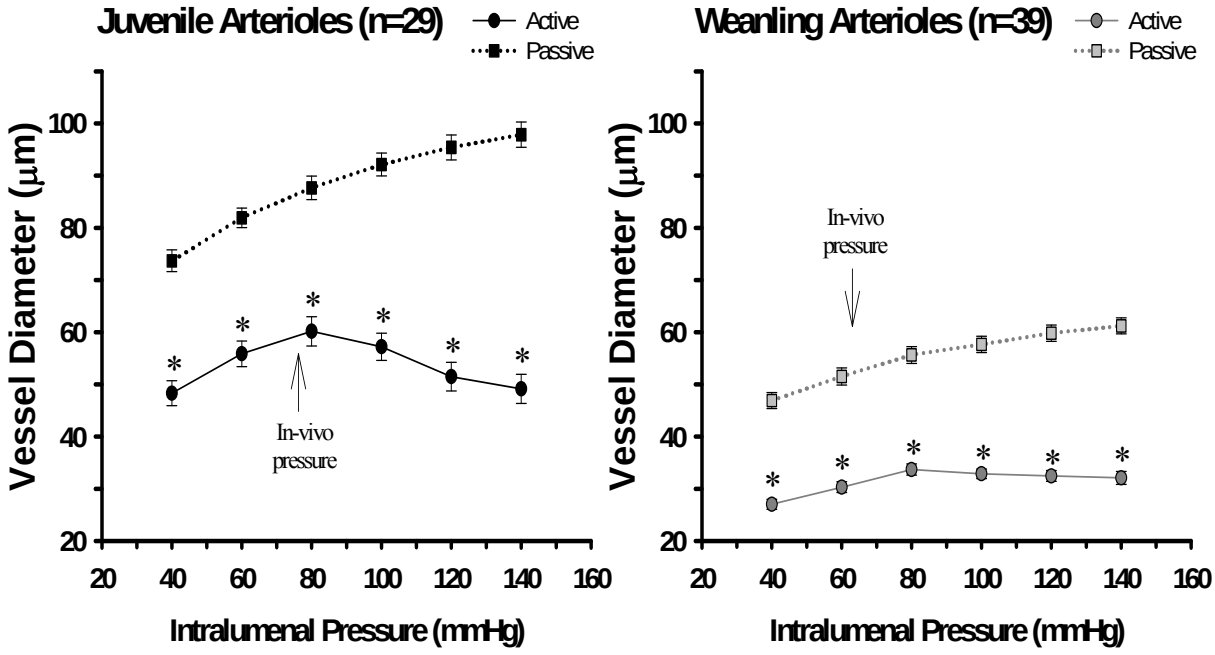
The HO inhibitor CrMP had no effect on steady state diameters or the myogenic indices for either juvenile or weanling arterioles (Figure 5).

Table 1: General characteristics of all rats and vessels used in this study

Animal Characteristics	**Weanlings**	**Juveniles**
N	39	29
Age (days)	26.6 ± 0.3	42.4 ± 0.4 *
Body Weight (g)	62 ± 1	173 ± 3 *
MAP (mmHg)	77 ± 2	96 ± 2 *
Vessel Characteristics (at lumenal pressure = 0.8 x MAP)		
n	39	29
Resting Diameter (µm)	34 ± 1	60 ± 3 *
Passive Diameter (µm)	56 ± 2	88 ± 2 *
Resting Vascular Tone (%)	38 ± 2	31 ± 3 *

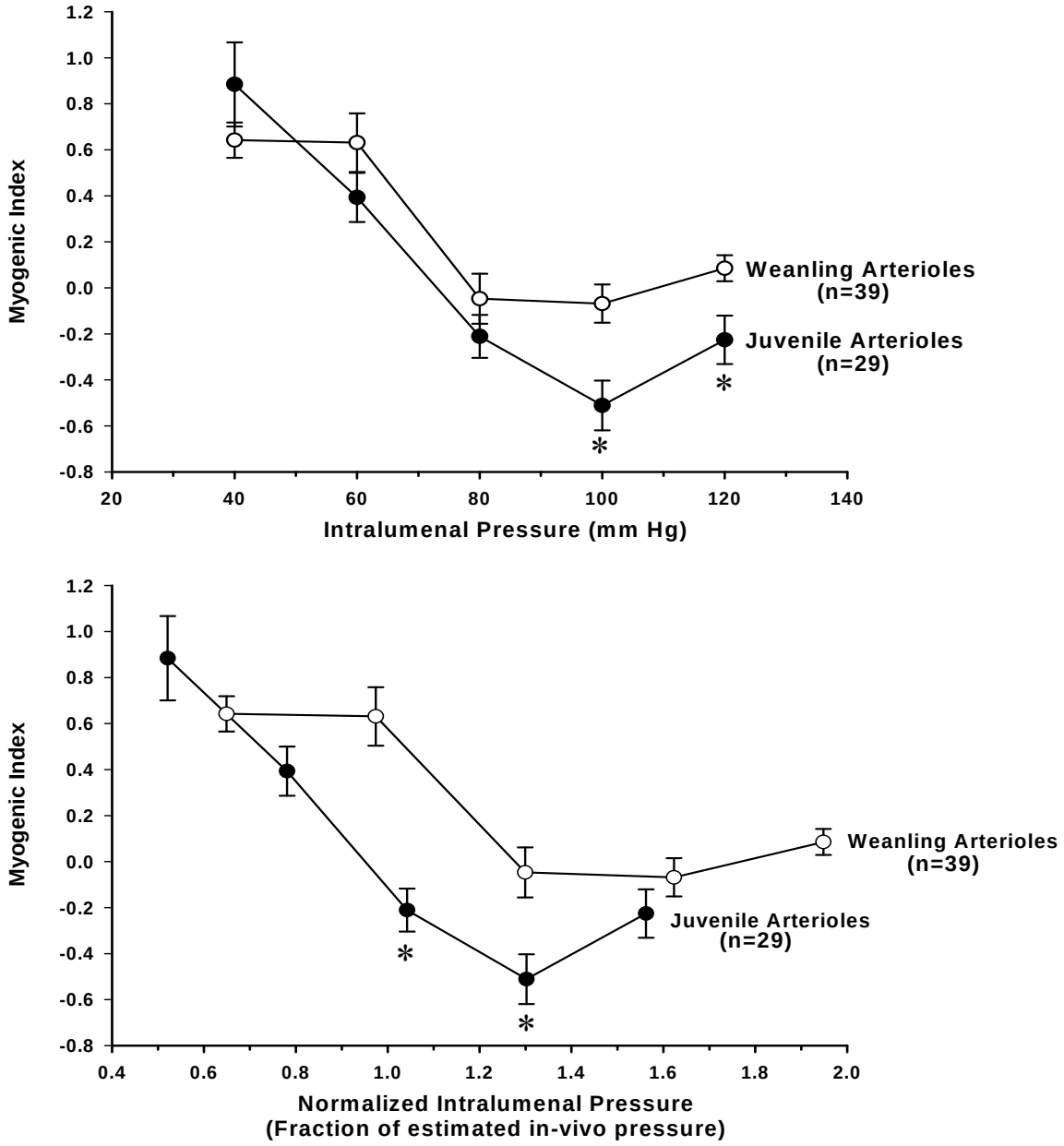
MAP = mean arterial pressure. Values are given as means ± SE. * $p<0.05$ vs. Weanling group.

Figure 1: Active and passive diameters as a function of intralumenal pressure in juvenile and weanling gracilis muscle arterioles



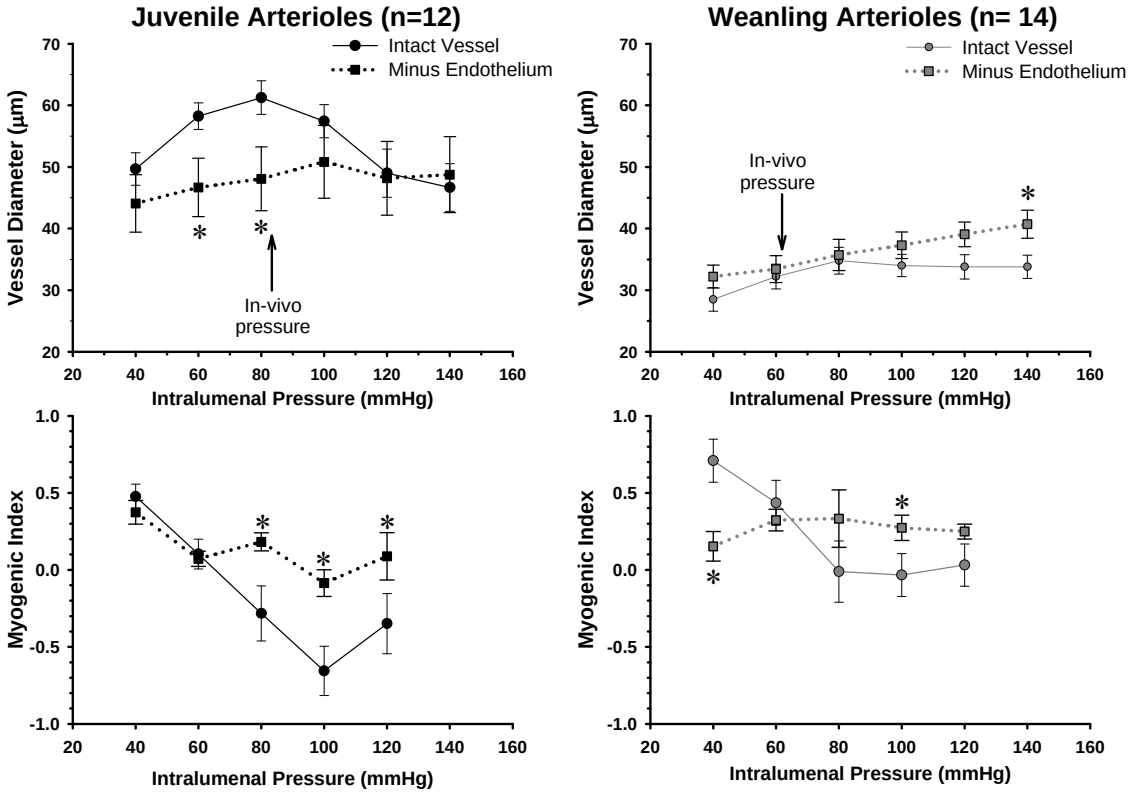
Values are given as means ± SE. *$p<0.05$ vs. Passive Diameter.

Figure 2: Calculated myogenic indices of juvenile and weanling arterioles as a function of absolute intralumenal pressure and as a function of intralumenal pressure normalized to the estimated *in vivo* arteriolar pressure for that age group.



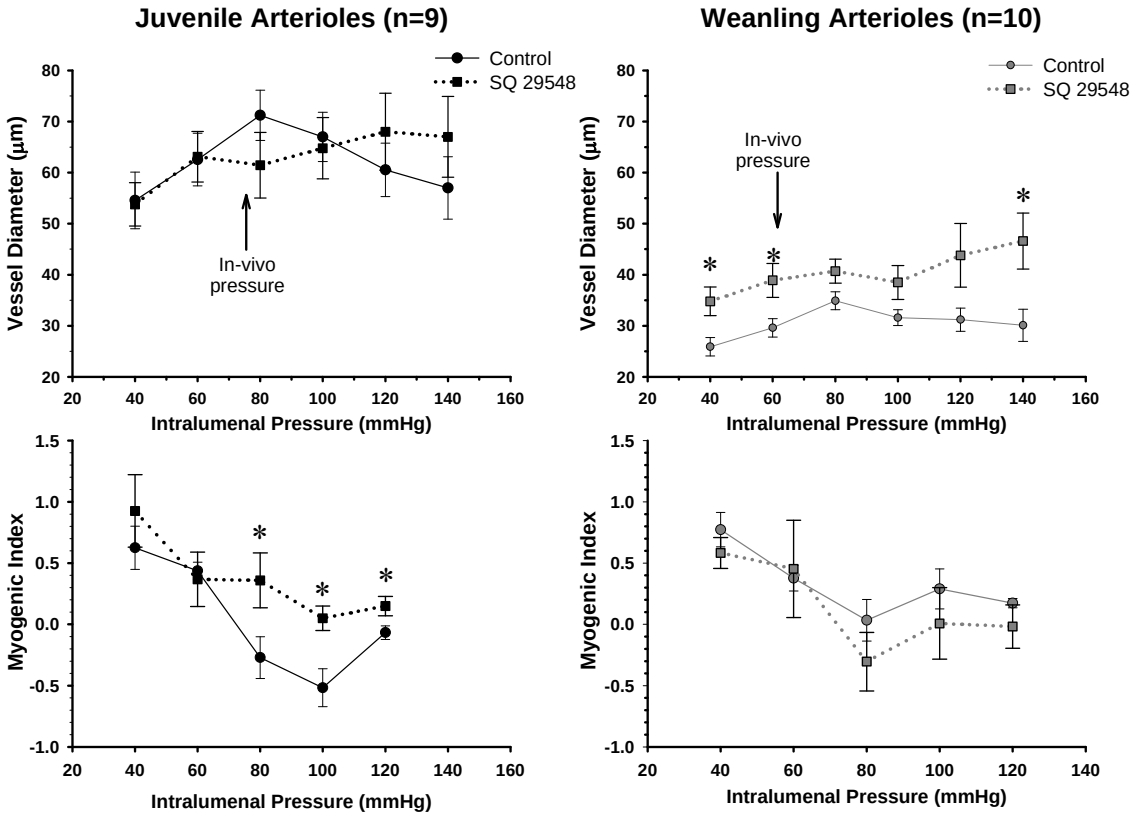
Values are given as means ± SE. n = number of vessels. *p<0.05 vs. Weanling Arterioles.

Figure 3: Effect of endothelial removal on steady-state diameters and myogenic indices over entire pressure range for juvenile and weanling arterioles



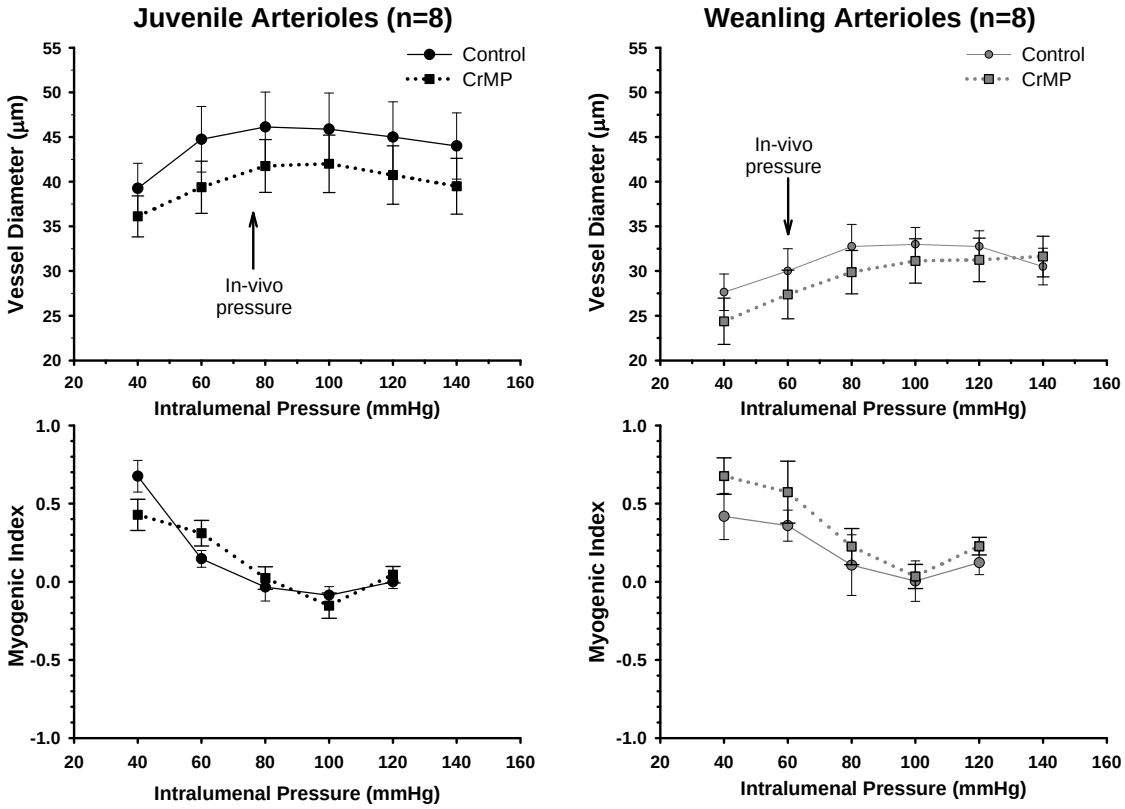
n = number of vessels. *p<0.05 vs. Intact Vessel.

Figure 4: Effect of SQ 29548 treatment on steady-state diameter and myogenic indices over entire pressure range for juvenile and weanling arterioles



n = number of vessels. *p<0.05 vs. Control.

Figure 5: Steady-state diameters and myogenic indices over entire pressure range before and after treatment with the heme oxygenase inhibitor chromium (III) mesoporphyrin IX chloride (CrMP) for juvenile and weanling arterioles.



n = number of vessels.

Discussion

The myogenic mechanism assists in the establishment of basal vascular tone, as well as in the autoregulation of blood flow and capillary hydrostatic pressure (Davis & Hill, 1999). During rapid postnatal growth, autoregulatory mechanisms may play a crucial role in maintaining sufficient blood flow to growing tissues. Investigators have found that myogenic responses of mesenteric arteries from 1-day old piglets are significantly greater than those from 10-day old piglets (Su *et al.*, 2003). Furthermore, the spontaneous tone developed by pressurized cerebral arteries from newborn mice is greater than that developed by those from 6-8 week-old mice over a range of intravascular pressures (Geary *et al.*, 2003). Consistent with these findings, we recently found that gracilis muscle arterioles isolated from weanling rats develop a significantly higher level of resting tone than those isolated from juvenile rats when equilibrated at their respective *in vivo* pressures (Balch Samora *et al.*, 2007a). Therefore, we undertook the current study to more vigorously test the hypothesis that arteriolar smooth muscle responsiveness to myogenic stimuli is greater in the younger rats. As in our earlier study, we found a higher level of spontaneous tone in weanling arterioles than in juvenile arterioles (Table 1). However, our calculations of myogenic index suggest that if anything, weanling arterioles have a smaller myogenic gain than juvenile arterioles when studied at their respective *in vivo* pressures, and also at pressures of 120 and 140 mmHg (Figure 2).

Because growth-related changes in arteriolar endothelial function have been reported by our laboratory and others (Linderman & Boegehold, 1999; Willis & Leffler, 1999; Nurkiewicz & Boegehold, 2004; Balch Samora *et al.*, 2007a), and because factors

released from the vascular endothelium can modulate smooth muscle myogenic tone (Hill *et al.*, 1990; Kuo *et al.*, 1991; Ekelund *et al.*, 1992; Sun *et al.*, 1995; de Wit *et al.*, 1998; Geary *et al.*, 2003), we examined the effects of endothelial denudation on the relationship between pressure and arteriolar diameter in both age groups. In juvenile arterioles, removal of the endothelium reduced steady-state diameters at pressures of 60 and 80 mmHg (Figure 3, top left panel), but this enhanced basal tone is apparently not myogenic in origin. This conclusion is based on our finding that after endothelial removal, the myogenic gain of these vessels is significantly *reduced* (i.e., a less negative myogenic index) at pressures from 80-120 mmHg (Figure 3, bottom left panel). This is not consistent with a previous report that endothelial removal has no effect on the myogenic index of gracilis muscle arterioles isolated from Wistar rats of the same age as our current juvenile group (Sun *et al.*, 1994). This may reflect differences in the strain of animal, such that even the same vessel under study may respond differently to changes in pressure. Supporting this theory is the finding of a consistent myogenic index from 60-120 mmHg in endothelial-intact 1st order arterioles in Sun's study, such that there is no change in slope, whereas in our study, from 40-100 mmHg, the myogenic index becomes progressively more negative, with a steep negative slope. However, it is also possible that the endothelium in the Wistar arterioles *may* contribute to myogenic activity, such that endothelium-derived vasoactive factors are released upon changes in pressure. However, when the vessels are denuded, all endothelium-derived substances (both constrictor and dilator factors) are removed, resulting in an overall consistent myogenic response that reflects only the smooth muscle contribution. This possibility was not tested in that study. Finally, this discrepancy may also be explained by differences in method of endothelial denudation, as Sun's group injected air into the lumen, whereas we

mechanically abraded the internal lumen. However, this explanation is less likely given the abolished response to ACh after denudation in the Wistar vessels. The reduction in the myogenic gain that we found after endothelial removal suggests that in the juvenile arterioles we studied, an endothelium-derived constrictor factor normally augments myogenic activity over a wide range of pressures. Application of SQ-29548 altered the myogenic index of juvenile arterioles in a manner similar to that seen with endothelial removal, i.e., a less negative index at pressures from 80-120 mmHg (Figure 4, bottom left panel). This finding is consistent with the possibility that PGH_2 or TXA_2 is the endothelial factor responsible for reinforcing the myogenic activity of these vessels. Other investigators have found a similar role for endothelium-derived constrictor prostanoids, but more typically in hypertensive rats (Huang & Koller, 1997; Ungvari & Koller, 2000). Ungvari and Koller (2000) determined that endothelium-derived endothelin and PGH_2/TxA_2 enhance arteriolar myogenic reactivity in hypertensive animals by increasing Ca^{2+} sensitivity of the vascular smooth muscle.

In contrast to our finding in juvenile arterioles, endothelial removal had no significant effect on the steady-state diameter of weanling arterioles, except for a modest dilation at 140 mmHg (Figure 3, top right panel), which is clearly above the physiological pressure range for these animals. Endothelial removal also had no consistent effect on the myogenic index of these vessels (Figure 3, bottom right panel). These observations suggest that an endothelial influence on the tone of weanling arterioles (myogenic or otherwise) arises in skeletal muscle sometime between 4 and 6 weeks of age. However, it is important to keep in mind that the arterioles in the present study were not being perfused, and therefore were not exposed to endothelial shear stress. In vivo, the release

(and therefore the influence) of endothelial dilator or constrictor factors at either age may be different than in the current situation.

TXA_2, a metabolite of PGH_2, is one of the most potent smooth muscle-contracting substances (Hamberg *et al.*, 1975). PGH_2/TXA_2 receptor antagonism tended to increase the steady-state diameter of weanling arterioles (Figure 4, top right panel), suggesting that basally-released PGH_2 or TXA_2 contributes to the tone of these vessels. However, this is apparently not through an effect on myogenic activity, since the myogenic index is unaltered by SQ-29548 (Figure 4, bottom right panel). In light of our finding that endothelial removal had no effect on the steady-state diameter of weanling arterioles, any PGH_2 or TXA_2 that influences the tone of these vessels must be of smooth muscle origin (Peredo & Enero, 1993; Cseko *et al.*, 2004).

Because we recently found a role for endogenous CO in mediating endothelium-dependent dilations in weanling and juvenile gracilis muscle arterioles, with exogenous CO at higher concentrations causing endothelium-independent constriction (unpublished observations), we reasoned that CO may also be playing a role in modulating myogenic reactivity. Based on their use of CrMP, Zhang et al (Zhang *et al.*, 2001) concluded that isolated gracilis muscle arterioles are capable of generating CO, and that this CO modulates myogenic vasoconstriction. However, in the present study, treatment with CrMP had no significant effect on myogenic responses in either group (Figure 5). Because the rats used in Zhang's study weighed 250-300 g, whereas our weanlings and juveniles weighed approximately 60 g and 170 g, respectively, a modulating role for CO in these responses may not arise until after adolescence.

Conclusion

These findings suggest that arteriolar growth during juvenile maturation is accompanied by an increase in myogenic responsiveness, possibly because endothelium-derived PGH_2 or TXA_2 assumes a role in reinforcing myogenic activity over this period. The results of this study further support the idea that alterations in the mechanisms of vascular tone regulation occur with growth and maturation, and that these evolving patterns may have significant implications for the regulation of tissue perfusion during microvascular development.

References

Andresen JJ, Shafi NI, Durante W & Bryan RM, Jr. (2006). Effects of carbon monoxide and heme oxygenase inhibitors in cerebral vessels of rats and mice. *Am J Physiol Heart Circ Physiol* 291, H223-230.

Aquin L, Sillau AH, Lechner AJ & Banchero N. (1980). Growth and skeletal muscle microvascularity in the guinea pig. *Microvascular research* 20, 41-50.

Balch Samora J, Frisbee J & Boegehold M. (2007a). Growth-dependent changes in endothelial factors regulating arteriolar tone. *American journal of physiology* 292, H207-H214.

Balch Samora J, Frisbee JC & Boegehold MA. (2007b). Hydrogen peroxide emerges as a regulator of arteriolar tone during microvascular network growth. *Faseb J* 21, A 494.

Cseko C, Bagi Z & Koller A. (2004). Biphasic effect of hydrogen peroxide on skeletal muscle arteriolar tone via activation of endothelial and smooth muscle signaling pathways. *J Appl Physiol* 97, 1130-1137.

Davis MJ. (1993). Myogenic response gradient in an arteriolar network. *The American journal of physiology* 264, H2168-2179.

Davis MJ & Hill MA. (1999). Signaling mechanisms underlying the vascular myogenic response. *Physiol Rev* 79, 387-423.

de Wit C, Jahrbeck B, Schafer C, Bolz SS & Pohl U. (1998). Nitric oxide opposes myogenic pressure responses predominantly in large arterioles in vivo. *Hypertension* 31, 787-794.

DeLano FA, Schmid-Schonbein GW, Skalak TC & Zweifach BW. (1991). Penetration of the systemic blood pressure into the microvasculature of rat skeletal muscle. *Microvascular research* 41, 92-110.

Ekelund U, Bjornberg J, Grande PO, Albert U & Mellander S. (1992). Myogenic vascular regulation in skeletal muscle in vivo is not dependent of endothelium-derived nitric oxide. *Acta physiologica Scandinavica* 144, 199-207.

Fredricks KT, Liu Y & Lombard JH. (1994). Response of extraparenchymal resistance arteries of rat skeletal muscle to reduced PO2. *The American journal of physiology* 267, H706-715.

Geary GG, Buchholz JN & Pearce WJ. (2003). Maturation depresses mouse cerebrovascular tone through endothelium-dependent mechanisms. *Am J Physiol Regul Integr Comp Physiol* 284, R734-741.

Hamberg M, Svensson J & Samuelsson B. (1975). Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. *Proceedings of the National Academy of Sciences of the United States of America* 72, 2994-2998.

Harder DR. (1987). Pressure-induced myogenic activation of cat cerebral arteries is dependent on intact endothelium. *Circulation research* 60, 102-107.

Hill MA, Davis MJ & Meininger GA. (1990). Cyclooxygenase inhibition potentiates myogenic activity in skeletal muscle arterioles. *The American journal of physiology* 258, H127-133.

Huang A & Koller A. (1997). Endothelin and prostaglandin H2 enhance arteriolar myogenic tone in hypertension. *Hypertension* 30, 1210-1215.

Huang A, Sun D & Koller A. (1993). Endothelial dysfunction augments myogenic arteriolar constriction in hypertension. *Hypertension* 22, 913-921.

Hudlicka O. (1985). Development and adaptability of microvasculature in skeletal muscle. *The Journal of experimental biology* 115, 215-228.

Katusic ZS, Shepherd JT & Vanhoutte PM. (1987). Endothelium-dependent contraction to stretch in canine basilar arteries. *The American journal of physiology* 252, H671-673.

Kelly AM, Rosser BW, Hoffman R, Panettieri RA, Schiaffino S, Rubinstein NA & Nemeth PM. (1991). Metabolic and contractile protein expression in developing rat diaphragm muscle. *J Neurosci* 11, 1231-1242.

Krijgsveld KL, Olson JM & Ricklefs RE. (2001). Catabolic capacity of the muscles of shorebird chicks: maturation of function in relation to body size. *Physiol Biochem Zool* 74, 250-260.

Kuo L, Chilian WM & Davis MJ. (1991). Interaction of pressure- and flow-induced responses in porcine coronary resistance vessels. *The American journal of physiology* 261, H1706-1715.

Linderman JR & Boegehold MA. (1996). Arteriolar network growth in rat striated muscle during juvenile maturation. *International journal of microcirculation, clinical and experimental / sponsored by the European Society for Microcirculation* 16, 232-239.

Linderman JR & Boegehold MA. (1998). Modulation of arteriolar sympathetic constriction by local nitric oxide: onset during rapid juvenile growth. *Microvascular research* 56, 192-202.

Linderman JR & Boegehold MA. (1999). Growth-related changes in the influence of nitric oxide on arteriolar tone. *The American journal of physiology* 277, H1570-1578.

Nurkiewicz TR & Boegehold MA. (2004). Calcium-independent release of endothelial nitric oxide in the arteriolar network: onset during rapid juvenile growth. *Microcirculation* 11, 453-462.

Peredo HA & Enero MA. (1993). Effect of endothelium removal on basal and muscarinic cholinergic stimulated rat mesenteric vascular bed prostanoid synthesis. *Prostaglandins, leukotrienes, and essential fatty acids* 48, 373-378.

Rubanyi GM. (1988). Endothelium-dependent pressure-induced contraction of isolated canine carotid arteries. *The American journal of physiology* 255, H783-788.

Sarelius IH, Damon DN & Duling BR. (1981). Microvascular adaptations during maturation of striated muscle. *The American journal of physiology* 241, H317-324.

Su BY, Reber KM, Nankervis CA & Nowicki PT. (2003). Development of the myogenic response in postnatal intestine: role of PKC. *Am J Physiol Gastrointest Liver Physiol* 284, G445-452.

Sun D, Huang A, Koller A & Kaley G. (1995). Flow-dependent dilation and myogenic constriction interact to establish the resistance of skeletal muscle arterioles. *Microcirculation* 2, 289-295.

Sun D, Kaley G & Koller A. (1994). Characteristics and origin of myogenic response in isolated gracilis muscle arterioles. *The American journal of physiology* 266, H1177-1183.

Uluoglu C & Zengil H. (2003). Comparison of different de-endothelization procedures in the isolated rat thoracic aorta: a short communication. *Research communications in molecular pathology and pharmacology* 113-114, 289-297.

Ungvari Z & Koller A. (2000). Endothelin and prostaglandin H(2)/thromboxane A(2) enhance myogenic constriction in hypertension by increasing Ca(2+) sensitivity of arteriolar smooth muscle. *Hypertension* 36, 856-861.

Wang DH & Prewitt RL. (1991). Microvascular development during normal growth and reduced blood flow: introduction of a new model. *The American journal of physiology* 260, H1966-1972.

Willis AP & Leffler CW. (1999). NO and prostanoids: age dependence of hypercapniaand histamine-induced dilations of pig pial arterioles. *The American journal of physiology* 277, H299-307.

Zhang F, Kaide J, Wei Y, Jiang H, Yu C, Balazy M, Abraham NG, Wang W & Nasjletti A. (2001). Carbon monoxide produced by isolated arterioles attenuates pressure-induced vasoconstriction. *Am J Physiol Heart Circ Physiol* 281, H350-358.

Zweifach BW, Kovalcheck S, De Lano F & Chen P. (1981). Micropressure-flow relationships in a skeletal muscle of spontaneously hypertensive rats. *Hypertension* 3, 601-614.

VI. GENERAL DISCUSSION

The primary objective of this dissertation project was to determine how microvascular function (specifically, the regulation of arteriolar tone) changes with postnatal growth and development. We found that endothelium-dependent arteriolar dilation in juvenile rats appears to be mediated by the combined release of endothelial NO, vasodilator prostanoids, H_2O_2, and CO. CO was the only observed mediator of endothelium-dependent dilation in vessels from weanling rats, although there must be other unidentified mediators, as HO inhibition only blunted endothelium-dependent responses. We also found that vessel growth is accompanied by an increase in overall arteriolar myogenic responsiveness, most likely due to increase in the contribution of PGH_2/TXA_2 to arteriolar myogenic behavior.

There are some practical implications of our studies. We were able to more thoroughly determine some of the changing mechanisms of vascular function during postnatal growth and maturation. A thorough understanding of the normal changes in endothelial/ smooth muscle signaling pathways and vascular mechanics during postnatal growth may lead to a better understanding of the pathophysiological changes that can also occur over this time in some disease states.

The public health implications of these studies are important given rapidly increasing rates of hypertension, diabetes, and dyslipidemia among children and adolescents in the United States (Wyatt *et al.*, 2006). The vascular dysfunction associated with some cardiovascular diseases has been found to develop well before adulthood (Bohlen & Lobach, 1978; Zweifach *et al.*, 1981; Prewitt *et al.*, 1982; Bohlen,

1987; Kunert *et al.*, 2001), so that changes linked to the pathological process become superimposed on the changes in vascular function that normally occur with juvenile growth. Increased rates of cardiovascular disease result in bleak medical, social, and economic consequences and can even shorten the longevity of Americans (Wyatt *et al.*, 2006). Therefore, to more clearly elucidate the mechanisms of progressive vascular dysfunction under such circumstances, it is necessary to gain a deeper understanding of those changes in vascular function that accompany normal growth, which we have begun to achieve with these studies. A clearer understanding of how the endothelium and smooth muscle work together to translate biochemical signals into vascular responses during blood vessel growth may ultimately lead to the identification of new therapeutic targets for the endothelial dysfunction associated with cardiovascular diseases that can begin to develop well before adulthood.

There are several limitations of our studies. First and foremost, we were unable to uncover the reasons for these mechanistic changes during postnatal growth. There are multiple changes which occur during development which may contribute to such vascular modifications. The mechanisms responsible for controlling arteriolar resistance and blood flow are not fixed at birth, but instead undergo marked changes during microvascular growth. Although there is a progressive increase in microvascular pressure and volume flow during arteriolar network growth in skeletal muscle (Zweifach *et al.*, 1981; Wang & Prewitt, 1991; Linderman & Boegehold, 1998, 1999), flow-related shear stress actually decreases due to a proportionately greater increase in arteriolar diameters than in blood flow velocity (Linderman & Boegehold, 1998, 1999).

Not only are arteriolar, capillary and venular networks rapidly growing (Sarelius *et al.*, 1981; Unthank & Bohlen, 1988; Wang & Prewitt, 1991; Linderman & Boegehold, 1996), and microvascular wall mass increasing during this time (Bohlen & Lobach, 1978; Wang & Prewitt, 1991), but anatomical modifications such as increases in fiber cross-sectional area of muscles (Enesco & Puddy, 1964; Sillau & Banchero, 1977; Ripoll *et al.*, 1979; Aquin *et al.*, 1980), and increases in length, diameter and wall mass of arterioles (Wang & Prewitt, 1991) are also taking place. Other changes include a decline in capillary density (Sillau & Banchero, 1977; Ripoll *et al.*, 1979; Sarelius *et al.*, 1981), and modifications in local endothelial cell environment and function over time (Risau, 1995; Garlanda & Dejana, 1997).

Another limitation is that the majority of our studies were conducted on vessels isolated from Sprague Dawley rats. Although *in vitro* studies allow investigators to control multiple varibles and explore potential physiological pathways and mechanisms of action, they are generally oversimplifications of the *in vivo* environment. All parenchymal and neural influences as well as humoral and endocrine effects are eliminated, which does not accurately reflect the *in vivo* milieu; these changes essentially remove the manifold subtle interactions that occur within the live animal.

Potential Role of Androgens

There are several reports in the literature suggesting that androgens can affect vascular reactivity, although there are not consistent findings (Barrett-Connor, 1995; Alexandersen *et al.*, 1996; Plut *et al.*, 2002). Whereas observational studies have demonstrated an inverse relationship between endogenous testosterone levels and adverse

cardiovascular events (Barrett-Connor, 1995; Alexandersen *et al.*, 1996), androgen deficiency is associated with increased flow-mediated dilation of the brachial artery when compared with age-matched controls (Zitzmann *et al.*, 2002). In fact, vascular reactivity in the brachial artery is improved when older men with advanced prostate cancer are castrated when compared with age-matched and cancer controls (Herman *et al.*, 1997).

In one set of experiments, we explored the possibility that some of the observed mechanistic changes during arteriolar growth might have been initiated by alterations in androgen concentration. Sprague-Dawley rats undergo puberty around postnatal day 50 (Baker *et al.*, 1979; Sharp & La Regina, 1998; Krinke, 2000), and reach sexual maturity at ~90 days (Ariyaratne & Chamindrani Mendis-Handagama, 2000). The number of androgen receptors in rat Sertoli cells markedly increases from postnatal days 10-20 to 35-60, and descent of the testes occurs around days 30-60 (Krinke, 2000). At ages 28 days and beyond, hypertrophy and hyperplasia of adult Leydig cells occurs, resulting in increases in testosterone production per testis in vitro (Ariyaratne & Chamindrani Mendis-Handagama, 2000). Actually, adult Leydig cells begin to secrete significant amounts of testosterone only after Day 35. Serum FSH levels, measured by radioimmunoassays in male Sprague-Dawley rats, peak between days 30 and 45. A rise in LH as well as testosterone occurs from days 30 to sexual maturity (Lee *et al.*, 1975).

Given these extensive hormonal changes that are transpiring from the weanling (~22 days of age) to juvenile (~46 days) animal, it was imperative to test the hypothesis that androgens may be responsible for bringing about the vascular modifications. This was accomplished by castrating one group of rats at age 22 days. Briefly, aseptic technique, involving sterile instruments and gloves, and a surgeon's gown and mask, was

used to perform either orchidectomy or sham surgeries on weanling rats. Rats were given carprofen SQ immediately after anesthesia induction with isoflurane gas. At 46 days, the juvenile animals were sacrificed after removal of gracilis muscle arterioles for in vitro measurements. Arterioles from both groups were challenged with ACh and simvastatin alone, followed by treatment with either L-NAME, to inhibit NOS, indomethacin, to inhibit cyclooxygenase, or combined NOS/COX inhibition.

Much to our surprise, we found no difference in vascular reactivity between the sham and castrated animals, suggesting that the striking hormonal changes that occur during this time frame are not responsible for the age-linked changes in vascular responses, at least with respect to the contribution of NO and prostanoids to endothelium-dependent dilation.

Timeframe of Mechanistic Changes

Throughout all of our studies, we have observed that these functional transformations in endothelium-dependent dilation occur within a window of approximately 3 weeks, between ~22 days of age (weanling animals) and ~46 days (juvenile animals). In order to more closely pinpoint the period of change, we performed experiments on arterioles from ~35 day old animals. Briefly, challenge with ACh and simvastatin in the presence of L-NAME, indomethacin, or the combined treatment of L-NAME + indomethacin elicited the same response in vessels from the 35 day old animals as those from 46 day old animals, suggesting that the period of mechanistic change in the contribution of NO and dilator prostanoids to endothelium-dependent dilation may be as narrow as 13 days.

In Vivo Studies

Because vascular tone, blood flow, endothelial function, and tissue metabolism are all interrelated, it is essential to investigate changes during postnatal growth with an in vivo approach to better understand the functional consequences of these changes. Using isolated vessels, we previously found age-related differences in myogenic responses and in the endothelial influence on arteriolar tone. In addition to metabolic stimuli, changes in the release of endothelium-derived factors and myogenic tone are thought to contribute to reactive hyperemia (Wolin *et al.,* 1990; O'Leary *et al.*, 1994; Gryglewski *et al.*, 1995; Meredith *et al.*, 1996; Koller & Bagi, 2002; Koller & Bagi, 2004; Toth *et al.*, 2007). Reactive or postischemic hyperemia, also referred to as flow- or shear-induced dilation, is defined as the transient increase in organ blood flow that follows a brief period of occlusion and represents an integrated and multifactorial perfusion response. Because the ability to match blood flow to metabolic demand is essential during growth, it is important to assess differences in flow-mediated responses as animals mature.

To measure vascular responses after brief (30 s) and longer (3 minute) occlusions in weanling and juvenile rats, we isolated and cleaned the left external iliac artery immediately proximal to the origin of the femoral artery. A loose suture was placed around the proximal end of the iliac artery, and a microcirculation blood flow probe (0.7 PSB, Transonic, Ithaca, NY) was placed around the artery distal to the suture to monitor hindlimb perfusion during the experimental procedures. Vascular occlusion was accomplished by elevating the suture until blood flow was eliminated, as observed by the

flow probe reading. Lowering the suture restored perfusion through the artery. The subsequent hyperemic response was monitored, with time-to-peak recorded for each group, and blood flow normalized to hind limb weight for each animal.

Resting blood flow, normalized to tissue mass, in juvenile rats was less than in weanling rats (see Figure 1 below). This agrees with findings in male hamster cremaster muscle, where significant decreases in resting erythrocyte velocity and estimated blood flow were observed to occur with postnatal growth (Sarelius, *et al.,* 1981; Berg & Sarelius, 1996). Similarly, in rat spinotrapezius muscle, there is a 25% volume flow *increase* from weanling to juvenile rats, but a *decrease* in blood flow per gram of tissue (Linderman & Boegehold, 1999). This finding suggests that tissue metabolic demand or skeletal muscle oxidative metabolism may decrease during growth. In fact, a decrease in oxidative capacity has been shown to occur during development in chickens (Hudlicka *et al*., 1973; Hudlicka, 1985), rabbits and kittens (Cotter, 1975), and mice (De Luise & Harker, 1989).

Normalized peak flow after release of the 30 s occlusion was greater in juvenile rats than weanling rats with these values representing a 404 ± 10% increase above control flow in juvenile rats vs. a 164 ± 12% increase above control flow in weanling rats. In contrast, peak flows recorded after 3 m occlusions were not different between age groups (Figure 1). The amplitude and duration of reactive hyperemia in skeletal muscle are determined in part by the length of the ischemic period (Gentry & Johnson, 1972; Messina *et al.*, 1977; Bjornberg *et al.*, 1990; Koller & Kaley, 1990b; Toth *et al.*, 2007). The dilation that occurs post-occlusion has traditionally been considered to occur either by myogenic or metabolic mechanisms, or by a combination of the two in a synergistic

fashion (Shepherd, 1964). It has been suggested that myogenic regulatory mechanisms dominate during brief occlusions (up to 30 seconds), but metabolic influences mediate hyperemic responses after longer occlusions (Johnson *et al.*, 1976; Bjornberg *et al.*, 1990; Koller & Kaley, 1990b; Toth *et al.*, 2007). If shorter occlusions do trigger myogenic mechanisms rather than metabolic influences, the finding of a greater than two-fold increase over basal flow in the juvenile vessels compared with the weanling vessels after a 30 s occlusion suggests that as animals mature, myogenic responses are enhanced. However, in Study 4, we found that calculated myogenic indices were similar for weanling and juvenile arterioles at pressures between 40 and 80 mmHg. Nonetheless, we also determined that the myogenic indices were significantly more negative for juvenile arterioles at pressures of 100 and 120 mmHg, indicating a greater myogenic responsiveness at these higher pressures. While the myogenic relaxation is induced by decreased stretch, there are various metabolic mechanisms, such as decreases in O_2, or an accumulation of CO_2, H^+ ion, and other metabolites that may mediate postischemic hyperemia (Shepherd, 1964; Johnson *et al.*, 1976; Lombard & Duling, 1981; Sherwood, 2007; Toth *et al.*, 2007). Therefore, even though the response was similar in both groups after a 3 m occlusion, we cannot assert the presence of analogous metabolic influences throughout postnatal growth.

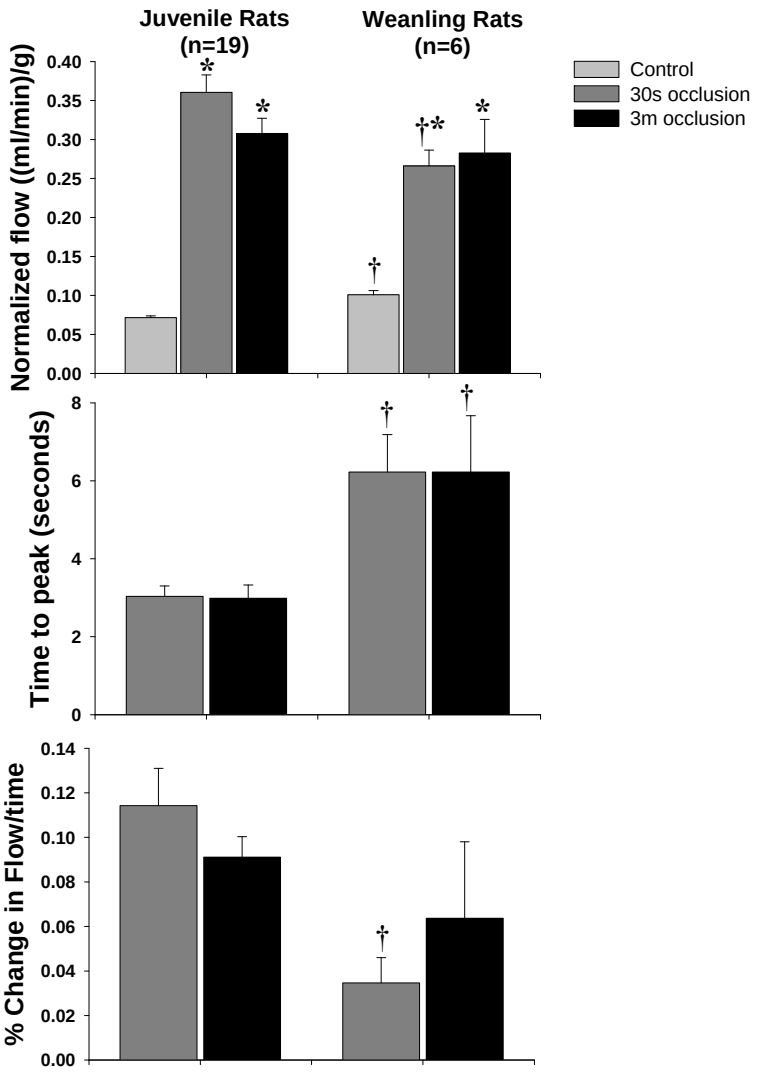
There are few investigations which have focused on the possible modulation of hyperemic responses with growth and development. Our findings differ from those observed in guinea pig coronary circulation, where postnatal growth is accompanied by a decrease in the peak hyperemic response following a 45-second occlusion (Toma *et al.,* 1985). Measurements of cutaneous blood flow in premature newborn infants (postnatal

age ranges from 0.3 to 72 days) after a one min occlusion indicate no influence of postconceptional age, postnatal age, weight, or transcutaneous O_2 tension on reactive hyperemic responses (Beaufort-Krol *et al.,* 1989). These discrepancies may be due to vascular bed or interspecies variability, or to differences in duration of occlusion, which affect amplitude (Gentry & Johnson, 1972; Messina *et al.*, 1977; Bjornberg *et al.*, 1990; Koller & Kaley, 1990b).

As also shown in Figure 1, the average time to reach the peak flow response also differed between groups, with weanling rats exhibiting a significantly slower response (an average time-to-peak that was more than twice that in the juvenile rats). This is somewhat surprising, since it has been argued that younger animals may have a superior capability for regulation of skeletal muscle blood flow (Proctor *et al.*, 1981). There could be many reasons for this finding, all of which are speculative. Because changes in tissue O_2 affect reactive hyperemic responses (Lombard & Duling, 1981), it is important to have an idea of what, if any, the differences in O_2 tension between groups might be. The increase in tissue blood flow after occlusion is essentially a "repayment" of "flow-debt" (Zatta & Headrick, 2005). Without having measured tissue O_2 concentrations, it is difficult to determine how much metabolic "debt" each animal sustained. Therefore, weanlings may not have needed as much functional recovery to return to metabolic balance. In an earlier study, whereas resting tissue PO_2 over a range of superfusate PO_2 values was found to be similar in 32, 60, and 80 day hamster cremaster muscle, in adult animals the tissue PO_2 decreased significantly during muscle stimulation, but was relatively unchanged in the younger animals (Proctor *et al.*, 1981). These studies by Proctor and colleagues suggest that there may, in fact, be maturational changes in O_2

utilization and response, such that an age-associated decrease in tissue O_2 consumption occurs.

Figure 1: Hindlimb blood flow in juvenile and weanling rats



Top panel: Hindlimb blood flow in juvenile and weanling rats under resting conditions, and peak values reached after relase of 30 second and 3 minute occlusions of the left external iliac artery. Middle panel: Time to peak flow value after occlusion release in both age groups. Bottom panel: % change in flow/time. n = number of animals. Values are given as means ± SE. * $p<0.05$ vs. Control. † $p<0.05$ vs. corresponding juvenile value.

VII. CONCLUSION

Because there are few studies that have investigated how postnatal growth affects microvascular function, we undertook these experiments, in part, to help fill a critical gap in the literature. Specifically, we wanted to gain further insight into growth-related changes in the control of skeletal muscle arterioles.

We found that at any given time, the tone of skeletal muscle arterioles does not reflect a single mechanism, but rather an integrated effect of multiple mechanisms that, to some extent, may compensate for one another should one pathway become compromised. During early microvascular growth, endothelium-dependent dilation appears to rely on CO, but not prostanoids, NO, H_2O_2, cytochrome P-450 metabolites, or any other EDHF. As animals mature, more vascular reliance on NO, dilator prostanoids, H_2O_2 and K^+ channels occurs. Arteriolar growth during juvenile maturation is also accompanied by an increase in myogenic responsiveness, possibly because endothelium-derived PGH_2 or TXA_2 assumes a role in reinforcing myogenic activity over this period. *In vivo* experiments demonstrated that although significant decreases in resting blood flow occur with growth, peak flow after release of short occlusions is significantly enhanced with growth and development.

Further investigation is needed in order to determine the extent to which changes in the control of skeletal muscle arterioles occurs with growth and maturation. A clearer understanding of how the endothelium and smooth muscle work together to translate biochemical signals into vascular responses during blood vessel growth may ultimately

lead to the identification of new therapeutic targets for the endothelial dysfunction associated with cardiovascular diseases that can begin to develop well before adulthood.

VIII. REFERENCES

Abid MR, Tsai JC, Spokes KC, Deshpande SS, Irani K & Aird WC. (2001). Vascular endothelial growth factor induces manganese-superoxide dismutase expression in endothelial cells by a Rac1-regulated NADPH oxidase-dependent mechanism. *Faseb J* 15, 2548-2550.

Adair TH, Gay WJ & Montani JP. (1990). Growth regulation of the vascular system: evidence for a metabolic hypothesis. *The American journal of physiology* 259, R393-404.

Agewall S, Henareh L, & Kublickiene K. (2006). Endothelial function in conduit and resistance arteries in men with coronary disease. *Atherosclerosis* 184 (1), 30-136.

Al-Mehdi AB, Zhao G, Dodia C, Tozawa K, Costa K, Muzykantov V, Ross C, Blecha F, Dinauer M & Fisher AB. (1998). Endothelial NADPH oxidase as the source of oxidants in lungs exposed to ischemia or high K^+. *Circulation research* 83, 730-737.

Alexandersen P, Haarbo J & Christiansen C. (1996). The relationship of natural androgens to coronary heart disease in males: a review. *Atherosclerosis* 125, 1-13.

Ames BN, Shigenaga MK & Hagen TM. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proceedings of the National Academy of Sciences of the United States of America* 90, 7915-7922.

Andree P, Dallner G & Ernster L. (1999). An Endogenous Lipid-Soluble Antioxidant in Animal Tissues. In *Reactive Oxygen Species in Biological Systems*, ed. Gilbert DL & Colton CA. Kluwer Academic/ Plenum Publishers, New York.

Anderson TJ, Uehata A, Gerhard MD et al. (1995). Close relation of endothelial function in the human coronary and peripheral circulations. *J Am Coll Cardiol* 26, 1235-1241.

Andresen JJ, Shafi NI, Durante W & Bryan RM, Jr. (2006). Effects of carbon monoxide and heme oxygenase inhibitors in cerebral vessels of rats and mice. *Am J Physiol Heart Circ Physiol* 291, H223-230.

Anrep G. (1912). On local vascular reactions and their interpretation. *J Physiol London* 45, 318-327.

Appleton SD, Chretien ML, McLaughlin BE, Vreman HJ, Stevenson DK, Brien JF, Nakatsu K, Maurice DH & Marks GS. (1999). Selective inhibition of heme oxygenase, without inhibition of nitric oxide synthase or soluble guanylyl cyclase,

by metalloporphyrins at low concentrations. *Drug metabolism and disposition: the biological fate of chemicals* 27, 1214-1219.

Aquin L, Sillau AH, Lechner AJ & Banchero N. (1980). Growth and skeletal muscle microvascularity in the guinea pig. *Microvascular research* 20, 41-50.

Archakov AI, Karuzina, II, Petushkova NA, Lisitsa AV & Zgoda VG. (2002). Production of carbon monoxide by cytochrome P450 during iron-dependent lipid peroxidation. *Toxicol In Vitro* 16, 1-10.

Ardanaz N & Pagano PJ. (2006). Hydrogen peroxide as a paracrine vascular mediator: regulation and signaling leading to dysfunction. *Experimental biology and medicine (Maywood, NJ* 231, 237-251.

Ariyaratne HB & Chamindrani Mendis-Handagama S. (2000). Changes in the testis interstitium of Sprague Dawley rats from birth to sexual maturity. *Biology of reproduction* 62, 680-690.

Aruoma OI & Halliwell B. (1987). Superoxide-dependent and ascorbate-dependent formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. Are lactoferrin and transferrin promoters of hydroxyl-radical generation? *The Biochemical journal* 241, 273-278.

Aruoma OI, Halliwell B, Gajewski E & Dizdaroglu M. (1991). Copper-ion-dependent damage to the bases in DNA in the presence of hydrogen peroxide. *The Biochemical journal* 273 (Pt 3), 601-604.

Ayajiki K, Kindermann M, Hecker M, Fleming I & Busse R. (1996). Intracellular pH and tyrosine phosphorylation but not calcium determine shear stress-induced nitric oxide production in native endothelial cells. *Circulation research* 78, 750-758.

Babior BM. (1999). NADPH oxidase: an update. *Blood* 93, 1464-1476.

Baker HJ, Lindsey JR & Weisbroth SH. (1979). *The Laboratory Rat*, vol. I. Academic Press, New York.

Bannister JV, Bannister WH & Rotilio G. (1987). Aspects of the structure, function, and applications of superoxide dismutase. *CRC critical reviews in biochemistry* 22, 111-180.

Barlow RS & White RE. (1998). Hydrogen peroxide relaxes porcine coronary arteries by stimulating BK_{Ca} channel activity. *The American journal of physiology* 275, H1283-1289.

Barrett-Connor EL. (1995). Testosterone and risk factors for cardiovascular disease in men. *Diabete & metabolisme* 21, 156-161.

Bayliss W. (1902). On the local reaction of the arterial wall to changes of internal pressure. *J Physiol London* 28, 220-231.

Beaufort-Krol GC, Suichies HE, Aarnoudse JG, Okken A, Jentink HW & Greve J. (1989). Postocclusive reactive hyperaemia of cutaneous blood flow in premature newborn infants. *Acta paediatrica Scandinavica* 360, 20-25.

Belhassen L, Pelle G, Sediame S et al. (2001). Endothelial dysfunction in patients with sickle cell disease is related to selective impairment of shear stress-mediated vasodilation. *Blood* 97, 1584-1589.

Berg BR & Sarelius IH. (1995). Functional capillary organization in striated muscle. *The American journal of physiology* 268, H1215-1222.

Berg BR & Sarelius IH. (1996). Erythrocyte flux in capillary networks during maturation: implications for oxygen delivery. *The American journal of physiology* 271, H2263-2273.

Bevan JA. (1987). Control of peripheral vascular resistance: evidence based on the in vitro study of resistance arteries. *Clinical and investigative medicine* 10, 568-572.

Beverelli F, Bea ML, Puybasset L, Giudicelli JF & Berdeaux A. (1997). Chronic inhibition of NO synthase enhances the production of prostacyclin in coronary arteries through upregulation of the cyclooxygenase type 1 isoform. *Fundamental & clinical pharmacology* 11, 252-259.

Bjornberg J, Albert U & Mellander S. (1990). Resistance responses in proximal arterial vessels, arterioles and veins during reactive hyperaemia in skeletal muscle and their underlying regulatory mechanisms. *Acta physiologica Scandinavica* 139, 535-550.

Boegehold MA. (1992). Reduced influence of nitric oxide on arteriolar tone in hypertensive Dahl rats. *Hypertension* 19, 290-295.

Boehning D, Moon C, Sharma S, Hurt KJ, Hester LD, Ronnett GV, Shugar D & Snyder SH. (2003). Carbon monoxide neurotransmission activated by CK2 phosphorylation of heme oxygenase-2. *Neuron* 40, 129-137.

Bohlen HG. (1987). Enhanced cerebral vascular regulation occurs by age 4 to 5 weeks in spontaneously hypertensive rats. *Hypertension* 9, 325-331.

Bohlen HG & Lobach D. (1978). In vivo study of microvascular wall characteristics and resting control in young and mature spontaneously hypertensive rats. *Blood vessels* 15, 322-330.

Bolotina VM, Najibi S, Palacino JJ, Pagano PJ & Cohen RA. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* 368, 850-853.

Bolz SS, de Wit C & Pohl U. (1999). Endothelium-derived hyperpolarizing factor but not NO reduces smooth muscle Ca^{2+} during acetylcholine-induced dilation of microvessels. *British journal of pharmacology* 128, 124-134.

Bouskela E & Wiederhielm CA. (1979). Microvascular myogenic reaction in the wing of the intact unanesthetized bat. *The American journal of physiology* 237, H59-65.

Boveris A, Martino E & Stoppani AO. (1977). Evaluation of the horseradish peroxidase-scopoletin method for the measurement of hydrogen peroxide formation in biological systems. *Analytical biochemistry* 80, 145-158.

Boveris A. (1984). Determination of the production of superoxide radicals and hydrogen peroxide in mitochondria. *Methods in enzymology* 105, 429-435.

Brayden JE. (2002). Functional roles of K_{ATP} channels in vascular smooth muscle. *Clinical and experimental pharmacology & physiology* 29, 312-316.

Brayden JE & Nelson MT. (1992). Regulation of arterial tone by activation of calcium-dependent potassium channels. *Science* 256, 532-535.

Bredt DS & Snyder SH. (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proceedings of the National Academy of Sciences of the United States of America* 87, 682-685.

Brian JE, Jr., Heistad DD & Faraci FM. (1994). Effect of carbon monoxide on rabbit cerebral arteries. *Stroke; a journal of cerebral circulation* 25, 639-643; discussion 643-634.

Brophy CM, Woodrum DA, Pollock J, Dickinson M, Komalavilas P, Cornwell TL & Lincoln TM. (2002). cGMP-dependent protein kinase expression restores contractile function in cultured vascular smooth muscle cells. *Journal of vascular research* 39, 95-103.

Brune B & Ullrich V. (1987). Inhibition of platelet aggregation by carbon monoxide is mediated by activation of guanylate cyclase. *Molecular pharmacology* 32, 497-504.

Burke-Wolin T, Abate CJ, Wolin MS & Gurtner GH. (1991). Hydrogen peroxide-induced pulmonary vasodilation: role of guanosine 3',5'-cyclic monophosphate. *The American journal of physiology* 261, L393-398.

Busse R, Fleming I & Hecker M. (1993). Signal transduction in endothelium-dependent vasodilatation. *European heart journal* 14 Suppl I, 2-9.

Busse R & Mulsch A. (1990). Calcium-dependent nitric oxide synthesis in endothelial cytosol is mediated by calmodulin. *FEBS letters* 265, 133-136.

Cadenas E. (1989). Biochemistry of oxygen toxicity. *Annual review of biochemistry* 58, 79-110.

Cadenas E & Davies KJ. (2000). Mitochondrial free radical generation, oxidative stress, and aging. *Free radical biology & medicine* 29, 222-230.

Cai H, Griendling KK & Harrison DG. (2003). The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases. *Trends in pharmacological sciences* 24, 471-478.

Cai H, Li Z, Dikalov S, Holland SM, Hwang J, Jo H, Dudley SC, Jr. & Harrison DG. (2002). NADPH oxidase-derived hydrogen peroxide mediates endothelial nitric oxide production in response to angiotensin II. *The Journal of biological chemistry* 277, 48311-48317.

Cai H & Harrison DG. (2000). Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circulation research* 87, 840-844.

Campbell WB & Gauthier KM. (2002). What is new in endothelium-derived hyperpolarizing factors? *Current opinion in nephrology and hypertension* 11, 177-183.

Campbell WB, Gebremedhin D, Pratt PF & Harder DR. (1996). Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circulation research* 78, 415-423.

Campbell WB & Harder DR. (1999). Endothelium-derived hyperpolarizing factors and vascular cytochrome P450 metabolites of arachidonic acid in the regulation of tone. *Circulation research* 84, 484-488.

Campbell WB & Falck JR. (2007). Arachidonic acid metabolites as endothelium-derived hyperpolarizing factors. *Hypertension* 49, 590-596.

Carrier GO, Fuchs LC, Winecoff AP, Giulumian AD & White RE. (1997). Nitrovasodilators relax mesenteric microvessels by cGMP-induced stimulation of Ca-activated K channels. *The American journal of physiology* 273, H76-84.

Carvajal JA, Germain AM, Huidobro-Toro JP & Weiner CP. (2000). Molecular mechanism of cGMP-mediated smooth muscle relaxation. *Journal of cellular physiology* 184, 409-420.

Cary SP & Marletta MA. (2001). The case of CO signaling: why the jury is still out. *The Journal of clinical investigation* 107, 1071-1073.

Cassin S, Dawes GS, Mott JC, Ross BB & Strang LB. (1964). The Vascular Resistance of the Foetal and Newly Ventilated Lung of the Lamb. *The Journal of physiology* 171, 61-79.

Chakder S, Rathi S, Ma XL & Rattan S. (1996). Heme oxygenase inhibitor zinc protoporphyrin IX causes an activation of nitric oxide synthase in the rabbit internal anal sphincter. *The Journal of pharmacology and experimental therapeutics* 277, 1376-1382.

Chen G, Suzuki H & Weston AH. (1988). Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels. *British journal of pharmacology* 95, 1165-1174.

Chen K, Thomas SR & Keaney JF, Jr. (2003). Beyond LDL oxidation: ROS in vascular signal transduction. *Free radical biology & medicine* 35, 117-132.

Chernick RJ, Martasek P, Levere RD, Margreiter R & Abraham NG. (1989). Sensitivity of human tissue heme oxygenase to a new synthetic metalloporphyrin. *Hepatology (Baltimore, Md* 10, 365-369.

Christodoulides N, Durante W, Kroll MH & Schafer AI. (1995). Vascular smooth muscle cell heme oxygenases generate guanylyl cyclase-stimulatory carbon monoxide. *Circulation* 91, 2306-2309.

Coburn RF, Blakemore WS & Forster RE. (1963). Endogenous carbon monoxide production in man. *The Journal of clinical investigation* 42, 1172-1178.

Coceani F. (1994). Control of the ductus arteriosus--a new function for cytochrome P450, endothelin and nitric oxide. *Biochemical pharmacology* 48, 1315-1318.

Coceani F, Kelsey L & Seidlitz E. (1996). Carbon monoxide-induced relaxation of the ductus arteriosus in the lamb: evidence against the prime role of guanylyl cyclase. *British journal of pharmacology* 118, 1689-1696.

Coceani F, Kelsey L, Seidlitz E, Marks GS, McLaughlin BE, Vreman HJ, Stevenson DK, Rabinovitch M & Ackerley C. (1997). Carbon monoxide formation in the ductus arteriosus in the lamb: implications for the regulation of muscle tone. *British journal of pharmacology* 120, 599-608.

Cohen G, Kim M & Ogwu V. (1996). A modified catalase assay suitable for a plate reader and for the analysis of brain cell cultures. *Journal of neuroscience methods* 67, 53-56.

Cohen RA & Vanhoutte PM. (1995). Endothelium-dependent hyperpolarization. Beyond nitric oxide and cyclic GMP. *Circulation* 92, 3337-3349.

Coleman HA, Tare M & Parkington HC. (2001). EDHF is not K^+ but may be due to spread of current from the endothelium in guinea pig arterioles. *American journal of physiology* 280, H2478-2483.

Collier J & Vallance P. (1989). Second messenger role for NO widens to nervous and immune systems. *Trends in pharmacological sciences* 10, 427-431.

Cornfield DN, Saqueton CB, Porter VA, Herron J, Resnik E, Haddad IY & Reeve HL. (2000). Voltage-gated K^+-channel activity in ovine pulmonary vasculature is developmentally regulated. *Am J Physiol Lung Cell Mol Physiol* 278, L1297-1304.

Cosentino F, Patton S, d'Uscio LV, Werner ER, Werner-Felmayer G, Moreau P, Malinski T & Luscher TF. (1998). Tetrahydrobiopterin alters superoxide and nitric oxide release in prehypertensive rats. *The Journal of clinical investigation* 101, 1530-1537.

Cotter MA. (1975). A study of the interrelationships between the vascular supply and metabolism of skeletal muscle fibres in the course of development and during chronic stimulation of adult muscles. Birmingham University, U.K.

Cruse I & Maines MD. (1988). Evidence suggesting that the two forms of heme oxygenase are products of different genes. *The Journal of biological chemistry* 263, 3348-3353.

Cseko C, Bagi Z & Koller A. (2004). Biphasic effect of hydrogen peroxide on skeletal muscle arteriolar tone via activation of endothelial and smooth muscle signaling pathways. *J Appl Physiol* 97, 1130-1137.

D'Angelo G & Meininger GA. (1994). Transduction mechanisms involved in the regulation of myogenic activity. *Hypertension* 23, 1096-1105.

Dai FX, Skopec J, Diederich A & Diederich D. (1992). Prostaglandin H_2 and thromboxane A_2 are contractile factors in intrarenal arteries of spontaneously hypertensive rats. *Hypertension* 19, 795-798.

Davidge ST, Baker PN, Laughlin MK & Roberts JM. (1995). Nitric oxide produced by endothelial cells increases production of eicosanoids through activation of prostaglandin H synthase. *Circulation research* 77, 274-283.

Davis MJ. (1993). Myogenic response gradient in an arteriolar network. *The American journal of physiology* 264, H2168-2179.

Davis MJ & Hill MA. (1999). Signaling mechanisms underlying the vascular myogenic response. *Physiol Rev* 79, 387-423.

Davis MJ, Meininger GA & Zawieja DC. (1992). Stretch-induced increases in intracellular calcium of isolated vascular smooth muscle cells. *The American journal of physiology* 263, H1292-1299.

Dawes GS, Mott JC, Widdicombe JG & Wyatt DG. (1953). Changes in the lungs of the new-born lamb. *The Journal of physiology* 121, 141-162.

Dawson JM & Hudlicka O. (1993). Can changes in microcirculation explain capillary growth in skeletal muscle? *International journal of experimental pathology* 74, 65-71.

De Luise M & Harker M. (1989). Skeletal muscle metabolism: effect of age, obesity, thyroid and nutritional status. *Hormone and metabolic research Hormon- und Stoffwechselforschung* 21, 410-415.

De Matteis F, Gibbs AH & Harvey C. (1985). Studies on the inhibition of ferrochelatase by N-alkylated dicarboxylic porphyrins. Steric factors involved and evidence that the inhibition is reversible. *The Biochemical journal* 226, 537-544.

DeMey J & Gray S. (1985). Endothelium-dependent reactivity in resistance vessels. *Prog appl microcirc* 8, 181-187.

Deskur E, Przywarska I, Dylewicz P, Szczesniak L, Rychlewski T, Wilk M & Wysocki H. (1998). Exercise-induced increase in hydrogen peroxide plasma levels is diminished by endurance training after myocardial infarction. *International journal of cardiology* 67, 219-224.

Dickinson DA & Forman HJ. (2002). Cellular glutathione and thiols metabolism. *Biochemical pharmacology* 64, 1019-1026.

Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R & Zeiher AM. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 399, 601-605.

Ding Y, McCoubrey WK, Jr. & Maines MD. (1999). Interaction of heme oxygenase-2 with nitric oxide donors. Is the oxygenase an intracellular 'sink' for NO? *European journal of biochemistry / FEBS* 264, 854-861.

Dora KA, Doyle MP, & Duling BR. (1997). Elevation of intracellular calcium in smooth muscle causes endothelial cell generation of NO in arterioles. *Proc Natl Acad Sci* 94, 6529-6534.

Droge W. (2001). Free radicals in the physiological control of cell function. *Physiol Rev* 82, 47-95.

Duling BR, Gore RW, Dacey RG, Jr. & Damon DN. (1981). Methods for isolation, cannulation, and in vitro study of single microvessels. *The American journal of physiology* 241, H108-116.

Dunn PM. (1999). UCL 1684: a potent blocker of Ca^{2+} -activated K^{+} channels in rat adrenal chromaffin cells in culture. *European journal of pharmacology* 368, 119-123.

Durante W, Kroll MH, Christodoulides N, Peyton KJ & Schafer AI. (1997). Nitric oxide induces heme oxygenase-1 gene expression and carbon monoxide production in vascular smooth muscle cells. *Circulation research* 80, 557-564.

Edwards G, Thollon C, Gardener MJ, Feletou M, Vilaine J, Vanhoutte PM & Weston AH. (2000). Role of gap junctions and EETs in endothelium-dependent hyperpolarization of porcine coronary artery. *British journal of pharmacology* 129, 1145-1154.

Emerson GG & Segal SS. (2000). Electrical coupling between endothelial cells and smooth muscle cells in hamster feed arteries: role in vasomotor control. *Circulation research* 87, 474-479.

Enesco M & Puddy D. (1964). Increase in the Number of Nuclei and Weight in Skeletal Muscle of Rats of Various Ages. *The American journal of anatomy* 114, 235-244.

Ewing JF, Raju VS & Maines MD. (1994). Induction of heart heme oxygenase-1 (HSP32) by hyperthermia: possible role in stress-mediated elevation of cyclic 3':5'-guanosine monophosphate. *The Journal of pharmacology and experimental therapeutics* 271, 408-414.

Falcone JC, Davis MJ & Meininger GA. (1991). Endothelial independence of myogenic response in isolated skeletal muscle arterioles. *The American journal of physiology* 260, H130-135.

Falcone JC, Granger HJ & Meininger GA. (1993). Enhanced myogenic activation in skeletal muscle arterioles from spontaneously hypertensive rats. *The American journal of physiology* 265, H1847-1855.

Fallet RW, Bast JP, Fujiwara K, Ishii N, Sansom SC & Carmines PK. (2001). Influence of Ca^{2+}-activated K^{+} channels on rat renal arteriolar responses to depolarizing agonists. *Am J Physiol Renal Physiol* 280, F583-591.

Fan B, Wang J, Stuehr DJ & Rousseau DL. (1997). NO synthase isozymes have distinct substrate binding sites. *Biochemistry* 36, 12660-12665.

Faraci FM & Didion SP. (2004). Vascular protection: superoxide dismutase isoforms in the vessel wall. *Arteriosclerosis, thrombosis, and vascular biology* 24, 1367-1373.

Favero TG, Zable AC & Abramson JJ. (1995). Hydrogen peroxide stimulates the Ca^{2+} release channel from skeletal muscle sarcoplasmic reticulum. *The Journal of biological chemistry* 270, 25557-25563.

Feletou M & Vanhoutte PM. (1988). Endothelium-dependent hyperpolarization of canine coronary smooth muscle. *British journal of pharmacology* 93, 515-524.

Feletou M & Vanhoutte PM. (1996). Endothelium-derived hyperpolarizing factor. *Clinical and experimental pharmacology & physiology* 23, 1082-1090.

Filipovic DM & Reeves WB. (1997). Hydrogen peroxide activates glibenclamide-sensitive K^+ channels in LLC-PK1 cells. *The American journal of physiology* 272, C737-743.

Finkel T & Holbrook NJ. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature* 408, 239-247.

Fisslthaler B, Dimmeler S, Hermann C, Busse R & Fleming I. (2000). Phosphorylation and activation of the endothelial nitric oxide synthase by fluid shear stress. *Acta physiologica Scandinavica* 168, 81-88.

Fleisch JH & Spaethe SM. (1981). Vasodilation and aging evaluated in the isolated perfused rat mesenteric vascular bed: preliminary observations on the vascular pharmacology of dobutamine. *Journal of cardiovascular pharmacology* 3, 187-196.

Fleming I. (2001). Cytochrome p450 and vascular homeostasis. *Circulation research* 89, 753-762.

Fleming I, Bauersachs J & Busse R. (1997). Calcium-dependent and calcium-independent activation of the endothelial NO synthase. *Journal of vascular research* 34, 165-174.

Fleming I & Busse R. (1999). Signal transduction of eNOS activation. *Cardiovascular research* 43, 532-541.

Folkow B. (1949). Intravascular pressure as a factor regulating the tone of the small vessels. *Acta Phys Scandinav* 17, 289-310.

Forstermann U, Boissel JP & Kleinert H. (1998). Expressional control of the 'constitutive' isoforms of nitric oxide synthase (NOS I and NOS III). *Faseb J* 12, 773-790.

Fridovich I. (1995). Superoxide radical and superoxide dismutases. *Annual review of biochemistry* 64, 97-112.

Frisbee JC. (2006). Vascular adrenergic tone and structural narrowing constrain reactive hyperemia in skeletal muscle of obese Zucker rats. *American journal of physiology* 290, H2066-2074.

Fujimoto S, Asano T, Sakai M, Sakurai K, Takagi D, Yoshimoto N & Itoh T. (2001). Mechanisms of hydrogen peroxide-induced relaxation in rabbit mesenteric small artery. *European journal of pharmacology* 412, 291-300.

Fukai T, Siegfried MR, Ushio-Fukai M, Griendling KK & Harrison DG. (1999). Modulation of extracellular superoxide dismutase expression by angiotensin II and hypertension. *Circulation research* 85, 23-28.

Funk CD. (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 294, 1871-1875.

Furchgott RF, Cherry PD, Zawadzki JV & Jothianandan D. (1984). Endothelial cells as mediators of vasodilation of arteries. *Journal of cardiovascular pharmacology* 6 Suppl 2, S336-343.

Furchgott RF & Jothianandan D. (1991). Endothelium-dependent and -independent vasodilation involving cyclic GMP: relaxation induced by nitric oxide, carbon monoxide and light. *Blood vessels* 28, 52-61.

Furchgott RF & Vanhoutte PM. (1989). Endothelium-derived relaxing and contracting factors. *Faseb J* 3, 2007-2018.

Furchgott RF & Zawadzki JV. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288, 373-376.

Galvez A, Gimenez-Gallego G, Reuben JP, Roy-Contancin L, Feigenbaum P, Kaczorowski GJ & Garcia ML. (1990). Purification and characterization of a unique, potent, peptidyl probe for the high conductance calcium-activated potassium channel from venom of the scorpion Buthus tamulus. *The Journal of biological chemistry* 265, 11083-11090.

Gao YJ & Lee RM. (2001). Hydrogen peroxide induces a greater contraction in mesenteric arteries of spontaneously hypertensive rats through thromboxane A_2 production. *British journal of pharmacology* 134, 1639-1646.

Garlanda C & Dejana E. (1997). Heterogeneity of endothelial cells. Specific markers. *Arteriosclerosis, thrombosis, and vascular biology* 17, 1193-1202.

Geary GG, Buchholz JN & Pearce WJ. (2003). Maturation depresses mouse cerebrovascular tone through endothelium-dependent mechanisms. *Am J Physiol Regul Integr Comp Physiol* 284, R734-741.

Gebremedhin D, Kaldunski M, Jacobs ER, Harder DR & Roman RJ. (1996). Coexistence of two types of Ca^{2+}-activated K^{+} channels in rat renal arterioles. *The American journal of physiology* 270, F69-81.

Gentry RM & Johnson PC. (1972). Reactive hyperemia in arterioles and capillaries of frog skeletal muscle following microocclusion. *Circulation research* 31, 953-965.

Gil-Longo J & Gonzalez-Vazquez C. (2005). Characterization of four different effects elicited by H_2O_2 in rat aorta. *Vascular pharmacology* 43, 128-138.

Gorlach A, Brandes RP, Nguyen K, Amidi M, Dehghani F & Busse R. (2000). A gp91phox containing NADPH oxidase selectively expressed in endothelial cells is a major source of oxygen radical generation in the arterial wall. *Circulation research* 87, 26-32.

Grehant N. (1894). *Les Gaz du Sang*. G. Masson, Paris.

Gribkoff VK, Starrett JE, Jr. & Dworetzky SI. (1997). The pharmacology and molecular biology of large-conductance calcium-activated (BK) potassium channels. *Advances in pharmacology (San Diego, Calif* 37, 319-348.

Griendling KK & Harrison DG. (1999). Dual role of reactive oxygen species in vascular growth. *Circulation research* 85, 562-563.

Griendling KK, Sorescu D, Lassegue B & Ushio-Fukai M. (2000a). Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. *Arteriosclerosis, thrombosis, and vascular biology* 20, 2175-2183.

Griendling KK, Sorescu D & Ushio-Fukai M. (2000b). NAD(P)H oxidase: role in cardiovascular biology and disease. *Circulation research* 86, 494-501.

Griffith OW & Stuehr DJ. (1995). Nitric oxide synthases: properties and catalytic mechanism. *Annual review of physiology* 57, 707-736.

Grover AK & Samson SE. (1988). Effect of superoxide radical on Ca^{2+} pumps of coronary artery. *The American journal of physiology* 255, C297-303.

Grover AK, Samson SE & Fomin VP. (1992). Peroxide inactivates calcium pumps in pig coronary artery. *The American journal of physiology* 263, H537-543.

Grozdanovic Z & Gossrau R. (1996). Expression of heme oxygenase-2 (HO-2)-like immunoreactivity in rat tissues. *Acta histochemica* 98, 203-214.

Grundemar L & Ny L. (1997). Pitfalls using metalloporphyrins in carbon monoxide research. *Trends in pharmacological sciences* 18, 193-195.

Gryglewski RJ, Botting RM & Vane JR. (1988). Mediators produced by the endothelial cell. *Hypertension* 12, 530-548.

Gryglewski RJ, Chlopicki S & Niezabitowski P. (1995). Endothelial control of coronary flow in perfused guinea pig heart. *Basic research in cardiology* 90, 119-124.

Gutteridge JM & Halliwell B. (1999). Antioxidant Protection and Oxygen Radical Signaling. In *Reactive Oxygen Species in Biological Systems*, ed. Gilbert DL & Colton CA. Kluwer Academic/Plenum Publishers, New York.

Gutteridge JM & Stocks J. (1981). Caeruloplasmin: physiological and pathological perspectives. *Critical reviews in clinical laboratory sciences* 14, 257-329.

Haddy FJ, Fleishman M & Emanuel DA. (1957). Effect of epinephrine, norepinephrine and serotonin upon systemic small and large vessel resistance. *Circulation research* 5, 247-251.

Halliwell B, Clement MV & Long LH. (2000). Hydrogen peroxide in the human body. *FEBS letters* 486, 10-13.

Halpern W, Osol G & Coy GS. (1984). Mechanical behavior of pressurized in vitro prearteriolar vessels determined with a video system. *Annals of biomedical engineering* 12, 463-479.

Harder DR. (1987). Pressure-induced myogenic activation of cat cerebral arteries is dependent on intact endothelium. *Circulation research* 60, 102-107.

Harder DR, Gilbert R & Lombard JH. (1987). Vascular muscle cell depolarization and activation in renal arteries on elevation of transmural pressure. *The American journal of physiology* 253, F778-781.

Harder DR, Narayanan J, Birks EK, Liard JF, Imig JD, Lombard JH, Lange AR & Roman RJ. (1996). Identification of a putative microvascular oxygen sensor. *Circulation research* 79, 54-61.

Harder DR, Lange AR, Gebremedhin D, Birks EK & Roman RJ. (1997). Cytochrome P450 metabolites of arachidonic acid as intracellular signaling molecules in vascular tissue. *Journal of vascular research* 34, 237-243.

Harlan JM, Levine JD, Callahan KS, Schwartz BR & Harker LA. (1984). Glutathione redox cycle protects cultured endothelial cells against lysis by extracellularly generated hydrogen peroxide. *The Journal of clinical investigation* 73, 706-713.

Harris ML, Schiller HJ, Reilly PM, Donowitz M, Grisham MB & Bulkley GB. (1992). Free radicals and other reactive oxygen metabolites in inflammatory bowel disease: cause, consequence or epiphenomenon? *Pharmacology & therapeutics* 53, 375-408.

Harrison R. (2002). Structure and function of xanthine oxidoreductase: where are we now? *Free radical biology & medicine* 33, 774-797.

Harrison R. (2004). Physiological roles of xanthine oxidoreductase. *Drug metabolism reviews* 36, 363-375.

Harvey W. (1628). *Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus*. Gvilielmi Fitzeri, Francofurti.

Hatton CJ, Carpenter E, Pepper DR, Kumar P & Peers C. (1997). Developmental changes in isolated rat type I carotid body cell K^+ currents and their modulation by hypoxia. *The Journal of physiology* 501 (Pt 1), 49-58.

Hemler ME, Cook HW & Lands WE. (1979). Prostaglandin biosynthesis can be triggered by lipid peroxides. *Archives of biochemistry and biophysics* 193, 340-345.

Herman SM, Robinson JT, McCredie RJ, Adams MR, Boyer MJ & Celermajer DS. (1997). Androgen deprivation is associated with enhanced endothelium-dependent dilatation in adult men. *Arteriosclerosis, thrombosis, and vascular biology* 17, 2004-2009.

Hester RL & Hammer LW. (2002). Venular-arteriolar communication in the regulation of blood flow. *Am J Physiol Regul Integr Comp Physiol* 282, R1280-1285.

Hill MA, Zou H, Potocnik SJ, Meininger GA & Davis MJ. (2001). Invited review: arteriolar smooth muscle mechanotransduction: Ca^{2+} signaling pathways underlying myogenic reactivity. *J Appl Physiol* 91, 973-983.

Hobbs AJ. (1997). Soluble guanylate cyclase: the forgotten sibling. *Trends in pharmacological sciences* 18, 484-491.

Hobbs AJ & Moncada S. (2003). Antiplatelet properties of a novel, non-NO-based soluble guanylate cyclase activator, BAY 41-2272. *Vascular pharmacology* 40, 149-154.

Hooker D. (1911). The chemical regulation of vascular tone as studied upon the perfused blood vessels of the frog. *The American journal of physiology* 28, 361-367.

Hoppeler H, Mathieu O, Weibel ER, Krauer R, Lindstedt SL & Taylor CR. (1981). Design of the mammalian respiratory system. VIII Capillaries in skeletal muscles. *Respiration physiology* 44, 129-150.

Horowitz A, Menice CB, Laporte R & Morgan KG. (1996). Mechanisms of smooth muscle contraction. *Physiol Rev* 76, 967-1003.

Houghton JL, Davison CA, Kuhner PA, Torossov MT, Strogatz DS, &Carr AA. (1998). Heterogeneous vasomotor responses of coronary conduit and resistance vessels in hypertension. *J Am Coll Cardiol* 31, 374-382.

Houston M, Estevez A, Chumley P, Aslan M, Marklund S, Parks DA & Freeman BA. (1999). Binding of xanthine oxidase to vascular endothelium. Kinetic characterization and oxidative impairment of nitric oxide-dependent signaling. *The Journal of biological chemistry* 274, 4985-4994.

Huang A & Koller A. (1996). Both nitric oxide and prostaglandin-mediated responses are impaired in skeletal muscle arterioles of hypertensive rats. *Journal of hypertension* 14, 887-895.

Huang A & Koller A. (1997). Endothelin and prostaglandin H_2 enhance arteriolar myogenic tone in hypertension. *Hypertension* 30, 1210-1215.

Huang A, Sun D & Koller A. (1993). Endothelial dysfunction augments myogenic arteriolar constriction in hypertension. *Hypertension* 22, 913-921.

Hudlicka O. (1969). Resting and postcontraction blood flow in slow and fast muscles of the chick during development. *Microvascular research* 1, 390-402.

Hudlicka O, Pette D & Staudte H. (1973). The relation between blood flow and enzymatic activities in slow and fast muscles during development. *Pflugers Arch* 343, 341-356.

Hudlicka O. (1985). Development and adaptability of microvasculature in skeletal muscle. *The Journal of experimental biology* 115, 215-228.

Hudlicka O & el Khelly F. (1985). Metabolic factors involved in regulation of muscle blood flow. *Journal of cardiovascular pharmacology* 7 Suppl 3, S59-72.

Hussain AS, Marks GS, Brien JF & Nakatsu K. (1997). The soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-alpha]quinoxalin-1-one (ODQ) inhibits relaxation of rabbit aortic rings induced by carbon monoxide, nitric oxide, and glyceryl trinitrate. *Canadian journal of physiology and pharmacology* 75, 1034-1037.

Hwa JJ & Bevan JA. (1986a). A nimodipine-resistant Ca^{2+} pathway is involved in myogenic tone in a resistance artery. *The American journal of physiology* 251, H182-189.

Hwa JJ & Bevan JA. (1986b). Stretch-dependent (myogenic) tone in rabbit ear resistance arteries. *The American journal of physiology* 250, H87-95.

Hyslop PA & Sklar LA. (1984). A quantitative fluorimetric assay for the determination of oxidant production by polymorphonuclear leukocytes: its use in the simultaneous fluorimetric assay of cellular activation processes. *Analytical biochemistry* 141, 280-286.

Ignarro LJ. (1993). Nitric oxide-mediated vasorelaxation. *Thrombosis and haemostasis* 70, 148-151.

Ignarro LJ, Buga GM, Wood KS, Byrns RE & Chaudhuri G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proceedings of the National Academy of Sciences of the United States of America* 84, 9265-9269.

Iida Y & Katusic ZS. (2000). Mechanisms of cerebral arterial relaxations to hydrogen peroxide. *Stroke; a journal of cerebral circulation* 31, 2224-2230.

Imai T, Morita T, Shindo T, Nagai R, Yazaki Y, Kurihara H, Suematsu M & Katayama S. (2001). Vascular smooth muscle cell-directed overexpression of heme oxygenase-1 elevates blood pressure through attenuation of nitric oxide-induced vasodilation in mice. *Circulation research* 89, 55-62.

Imig JD. (1999). Epoxyeicosatrienoic acids. Biosynthesis, regulation, and actions. *Methods in molecular biology (Clifton, NJ* 120, 173-192.

Imig JD, Inscho EW, Deichmann PC, Reddy KM, & Falck JR. (1999). Afferent arteriolar vasodilation to the sulfonimide analog of 11,12-epoxyeicosatrienoic acid involves protein kinase A. *Hypertension* 33, 408-413.

Inscho EW, Cook AK, Mui V & Imig JD. (1998). Calcium mobilization contributes to pressure-mediated afferent arteriolar vasoconstriction. *Hypertension* 31, 421-428.

Ishikawa M, Kajimura M, Adachi T, Maruyama K, Makino N, Goda N, Yamaguchi T, Sekizuka E & Suematsu M. (2005). Carbon monoxide from heme oxygenase-2 is a tonic regulator against NO-dependent vasodilatation in the adult rat cerebral microcirculation. *Circulation research* 97, e104-114.

Jackson PA & Duling BR. (1989). Myogenic response and wall mechanics of arterioles. *The American journal of physiology* 257, H1147-1155.

Jackson WF. (2000). Ion channels and vascular tone. *Hypertension* 35, 173-178.

Jackson WF. (2005). Potassium channels in the peripheral microcirculation. *Microcirculation* 12, 113-127.

Jaggar JH, Leffler CW, Cheranov SY, Tcheranova D, E S & Cheng X. (2002). Carbon monoxide dilates cerebral arterioles by enhancing the coupling of Ca^{2+} sparks to Ca^{2+}-activated K^{+} channels. *Circulation research* 91, 610-617.

Jernigan NL, O'Donaughy TL & Walker BR. (2001). Correlation of HO-1 expression with onset and reversal of hypoxia-induced vasoconstrictor hyporeactivity. *Am J Physiol Heart Circ Physiol* 281, H298-307.

Jin N & Rhoades RA. (1997). Activation of tyrosine kinases in H_2O_2-induced contraction in pulmonary artery. *The American journal of physiology* 272, H2686-2692.

Joannides R, Haefeli WE, Linder L, Richard V, Bakkali EH, Thuillez C & Luscher TF. (1995). Nitric oxide is responsible for flow-dependent dilatation of human peripheral conduit arteries in vivo. *Circulation* 91, 1314-1319.

Joannides R, Bellien J, & Thuillez C. (2006). Clinical methods for the evaluation of endothelial function - a focus on resistance arteries. *Fundamental & Clinical Pharmacology* 20 (3), 311–320.

Johnson FK, Durante W, Peyton KJ & Johnson RA. (2003). Heme oxygenase inhibitor restores arteriolar nitric oxide function in dahl rats. *Hypertension* 41, 149-155.

Johnson FK & Johnson RA. (2003). Carbon monoxide promotes endothelium-dependent constriction of isolated gracilis muscle arterioles. *American journal of physiology* 285, R536-541.

Johnson FK, Teran FJ, Prieto-Carrasquero M & Johnson RA. (2002). Vascular effects of a heme oxygenase inhibitor are enhanced in the absence of nitric oxide. *Am J Hypertens* 15, 1074-1080.

Johnson P. (1980). *The myogenic response. In: Handbook of Physiology. The Cardiovascular System. Vascular Smooth Muscle*, vol. II, sect. 2, p. 409-442. Am Physiol Soc, Bethesda, MD.

Johnson PC. (1964a). Origin, Localization, and Homeostatic Significance of Autoregulation in the Intestine. *Circulation research* 15, SUPPL:225-233.

Johnson PC. (1964b). Review of Previous Studies and Current Theories of Autoregulation. *Circulation research* 15, SUPPL:2-9.

Johnson PC, Burton KS, Henrich H & Henrich U. (1976). Effect of occlusion duration on reactive hyperemia in sartorius muscle capillaries. *The American journal of physiology* 230, 715-719.

Johnson RA, Kozma F & Colombari E. (1999). Carbon monoxide: from toxin to endogenous modulator of cardiovascular functions. *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica,* 32, 1-14.

Johnson RA, Lavesa M, Askari B, Abraham NG & Nasjletti A. (1995). A heme oxygenase product, presumably carbon monoxide, mediates a vasodepressor function in rats. *Hypertension* 25, 166-169.

Johnson RA, Lavesa M, DeSeyn K, Scholer MJ & Nasjletti A. (1996). Heme oxygenase substrates acutely lower blood pressure in hypertensive rats. *The American journal of physiology* 271, H1132-1138.

Kaide J, Zhang F & Nasjletti A. (2002). Carbon monoxide attenuates the nitric oxide-mediated component of the relaxing action of acetylcholine (Abstract). *Hypertension* 40, 403.

Kanatsuka H, Sekiguchi N, Sato K, Akai K, Wang Y, Komaru T, Ashikawa K & Takishima T. (1992). Microvascular sites and mechanisms responsible for reactive hyperemia in the coronary circulation of the beating canine heart. *Circulation research* 71, 912-922.

Karyakin AA, Puganova EA, Budashov IA, Kurochkin IN, Karyakina EE, Levchenko VA, Matveyenko VN & Varfolomeyev SD. (2004). Prussian blue based nanoelectrode arrays for H_2O_2 detection. *Analytical chemistry* 76, 474-478.

Katusic ZS. (1996). Superoxide anion and endothelial regulation of arterial tone. *Free radical biology & medicine* 20, 443-448.

Katusic ZS, Shepherd JT & Vanhoutte PM. (1987). Endothelium-dependent contraction to stretch in canine basilar arteries. *The American journal of physiology* 252, H671-673.

Keyse SM & Tyrrell RM. (1989). Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. *Proceedings of the National Academy of Sciences of the United States of America* 86, 99-103.

Kharitonov VG, Sharma VS, Pilz RB, Magde D & Koesling D. (1995). Basis of guanylate cyclase activation by carbon monoxide. *Proceedings of the National Academy of Sciences of the United States of America* 92, 2568-2571.

Kikuchi G & Hayashi N. (1981). Regulation by heme of synthesis and intracellular translocation of delta-aminolevulinate synthase in the liver. *Molecular and cellular biochemistry* 37, 27-41.

Kim HP, Ryter SW & Choi AM. (2006). CO as a cellular signaling molecule. *Annu Rev Pharmacol Toxicol* 46, 411-449.

Knot HJ & Nelson MT. (1995). Regulation of membrane potential and diameter by voltage-dependent K^+ channels in rabbit myogenic cerebral arteries. *The American journal of physiology* 269, H348-355.

Knot HJ & Nelson MT. (1998). Regulation of arterial diameter and wall [Ca^{2+}] in cerebral arteries of rat by membrane potential and intravascular pressure. *The Journal of physiology* 508 (Pt 1), 199-209.

Koga T, Takata Y, Kobayashi K, Fujii K, Nagao T & Fujishima M. (1992). Age-related changes in P_2-purinergic receptors on vascular smooth muscle and endothelium. *Hypertension* 19, 286-289.

Koga T, Takata Y, Kobayashi K, Takishita S, Yamashita Y & Fujishima M. (1989). Age and hypertension promote endothelium-dependent contractions to acetylcholine in the aorta of the rat. *Hypertension* 14, 542-548.

Kolber KA, Gao Y & Raj JU. (2000). Maturational changes in endothelium-derived nitric oxide-mediated relaxation of ovine pulmonary arteries. *Biology of the neonate* 77, 123-130.

Koller A & Bagi Z. (2002). On the role of mechanosensitive mechanisms eliciting reactive hyperemia. *American journal of physiology* 283, H2250-2259.

Koller A & Kaley G. (1990a). Prostaglandins mediate arteriolar dilation to increased blood flow velocity in skeletal muscle microcirculation. *Circulation research* 67, 529-534.

Koller A & Kaley G. (1990b). Role of endothelium in reactive dilation of skeletal muscle arterioles. *The American journal of physiology* 259, H1313-1316.

Koller A & Kaley G. (1991). Endothelial regulation of wall shear stress and blood flow in skeletal muscle microcirculation. *The American journal of physiology* 260, H862-868.

Korenaga R, Ando J, Ohtsuka A, Sakuma I, Yang W, Toyo-oka T & Kamiya A. (1993). Close correlation between cytoplasmic Ca^{2+} levels and release of an endothelium-derived relaxing factor from cultured endothelial cells. *Cell structure and function* 18, 95-104.

Korn SJ & Trapani JG. (2005). Potassium channels. *IEEE transactions on nanobioscience* 4, 21-33.

Kozma F, Johnson RA & Nasjletti A. (1997). Role of carbon monoxide in heme-induced vasodilation. *European journal of pharmacology* 323, R1-2.

Kozma F, Johnson RA, Zhang F, Yu C, Tong X & Nasjletti A. (1999). Contribution of endogenous carbon monoxide to regulation of diameter in resistance vessels. *The American journal of physiology* 276, R1087-1094.

Krinke GJ. (2000). *The Laboratory Rat*. Academic Press, Stein Switzerland.

Krummen S, Falck JR & Thorin E. (2005). Two distinct pathways account for EDHF-dependent dilatation in the gracilis artery of dyslipidaemic hApoB+/+ mice. *British journal of pharmacology* 145, 264-270.

Kubes P. (1995). Nitric oxide affects microvascular permeability in the intact and inflamed vasculature. *Microcirculation* 2, 235-244.

Kunert MP, Roman RJ, Falck JR & Lombard JH. (2001). Differential effect of cytochrome P-450 omega-hydroxylase inhibition on O_2-induced constriction of arterioles in SHR with early and established hypertension. *Microcirculation* 8, 435-443.

Kuo L, Chilian WM & Davis MJ. (1991). Interaction of pressure- and flow-induced responses in porcine coronary resistance vessels. *The American journal of physiology* 261, H1706-1715.

Kuo L, Davis MJ & Chilian WM. (1988). Myogenic activity in isolated subepicardial and subendocardial coronary arterioles. *The American journal of physiology* 255, H1558-1562.

Kuo L, Davis MJ & Chilian WM. (1990). Endothelium-dependent, flow-induced dilation of isolated coronary arterioles. *The American journal of physiology* 259, H1063-1070.

Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, Lefer DJ, Sessa WC & Walsh K. (2000). The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. *Nature medicine* 6, 1004-1010.

La M & Reid JJ. (1995). Endothelin-1 and the regulation of vascular tone. *Clinical and experimental pharmacology & physiology* 22, 315-323.

Lacy F, O'Connor DT & Schmid-Schonbein GW. (1998). Plasma hydrogen peroxide production in hypertensives and normotensive subjects at genetic risk of hypertension. *Journal of hypertension* 16, 291-303.

Laher I & Bevan JA. (1993). Stretch-dependent myogenic tone in isolated blood vessels. In *Mechanoreceptors by the Vascular Wall*, ed. Rubanyi GM. Futura Publishing Co., Inc., Mount Kisco, NY.

Lamas S, Marsden PA, Li GK, Tempst P & Michel T. (1992). Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. *Proceedings of the National Academy of Sciences of the United States of America* 89, 6348-6352.

Lassegue B & Clempus RE. (2003). Vascular NADPH oxidases: specific features, expression, and regulation. *Am J Physiol Regul Integr Comp Physiol* 285, R277-297.

Ledoux J, Werner ME, Brayden JE & Nelson MT. (2006). Calcium-activated potassium channels and the regulation of vascular tone. *Physiology (Bethesda, Md* 21, 69-78.

Lee VW, de Kretser DM, Hudson B & Wang C. (1975). Variations in serum FSH, LH and testosterone levels in male rats from birth to sexual maturity. *Journal of reproduction and fertility* 42, 121-126.

Leffler CW, Balabanova L, Fedinec AL & Parfenova H. (2005). Nitric oxide increases carbon monoxide production by piglet cerebral microvessels. *Am J Physiol Heart Circ Physiol* 289, H1442-1447.

Leffler CW, Balabanova L, Sullivan CD, Wang X, Fedinec AL & Parfenova H. (2003). Regulation of CO production in cerebral microvessels of newborn pigs. *Am J Physiol Heart Circ Physiol* 285, H292-297.

Leffler CW, Nasjletti A, Johnson RA & Fedinec AL. (2001). Contributions of prostacyclin and nitric oxide to carbon monoxide-induced cerebrovascular dilation in piglets. *American journal of physiology* 280, H1490-1495.

Leffler CW, Nasjletti A, Yu C, Johnson RA, Fedinec AL & Walker N. (1999). Carbon monoxide and cerebral microvascular tone in newborn pigs. *The American journal of physiology* 276, H1641-1646.

Lew MJ, Rivers RJ & Duling BR. (1989). Arteriolar smooth muscle responses are modulated by an intramural diffusion barrier. *The American journal of physiology* 257, H10-16.

Li F & Joshua IG. (1993). Decreased arteriolar endothelium-derived relaxing factor production during the development of genetic hypertension. *Clin Exp Hypertens* 15, 511-526.

Li JM & Shah AM. (2004). Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology. *Am J Physiol Regul Integr Comp Physiol* 287, R1014-1030.

Lim I, Gibbons SJ, Lyford GL, Miller SM, Strege PR, Sarr MG, Chatterjee S, Szurszewski JH, Shah VH & Farrugia G. (2005). Carbon monoxide activates human intestinal smooth muscle L-type Ca^{2+} channels through a nitric oxide-dependent mechanism. *Am J Physiol Gastrointest Liver Physiol* 288, G7-14.

Lin H & McGrath JJ. (1988). Vasodilating effects of carbon monoxide. *Drug and chemical toxicology* 11, 371-385.

Linderman JR & Boegehold MA. (1996). Arteriolar network growth in rat striated muscle during juvenile maturation. *International journal of microcirculation, clinical and experimental / sponsored by the European Society for Microcirculation* 16, 232-239.

Linderman JR & Boegehold MA. (1998). Modulation of arteriolar sympathetic constriction by local nitric oxide: onset during rapid juvenile growth. *Microvascular research* 56, 192-202.

Linderman JR & Boegehold MA. (1999). Growth-related changes in the influence of nitric oxide on arteriolar tone. *The American journal of physiology* 277, H1570-1578.

Lipton SA, Choi YB, Pan ZH, Lei SZ, Chen HS, Sucher NJ, Loscalzo J, Singel DJ & Stamler JS. (1993). A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* 364, 626-632.

Lister G, Walter TK, Versmold HT, Dallman PR & Rudolph AM. (1979). Oxygen delivery in lambs: cardiovascular and hematologic development. *The American journal of physiology* 237, H668-675.

Lombard JH & Duling BR. (1981). Multiple mechanisms of reactive hyperemia in arterioles of the hamster cheek pouch. *The American journal of physiology* 241, H748-755.

Lombard JH, Kunert MP, Roman RJ, Falck JR, Harder DR & Jackson WF. (1999a). Cytochrome P-450 omega-hydroxylase senses O_2 in hamster muscle, but not cheek pouch epithelium, microcirculation. *The American journal of physiology* 276, H503-508.

Lombard JH, Liu Y, Fredricks KT, Bizub DM, Roman RJ & Rusch NJ. (1999b). Electrical and mechanical responses of rat middle cerebral arteries to reduced PO_2 and prostacyclin. *The American journal of physiology* 276, H509-516.

Long W, Zhang L & Longo LD. (2000). Cerebral artery K_{ATP}- and K_{Ca}-channel activity and contractility: changes with development. *Am J Physiol Regul Integr Comp Physiol* 279, R2004-2014.

Lucchesi PA, Belmadani S & Matrougui K. (2005). Hydrogen peroxide acts as both vasodilator and vasoconstrictor in the control of perfused mouse mesenteric resistance arteries. *Journal of hypertension* 23, 571-579.

Luckhoff A, Pohl U, Mulsch A & Busse R. (1988). Differential role of extra- and intracellular calcium in the release of EDRF and prostacyclin from cultured endothelial cells. *British journal of pharmacology* 95, 189-196.

Luo D & Vincent SR. (1994). Metalloporphyrins inhibit nitric oxide-dependent cGMP formation in vivo. *European journal of pharmacology* 267, 263-267.

Mabanta L, Valane P, Borne J & Frame MD. (2006). Initiation of remote microvascular preconditioning requires K_{ATP} channel activity. *American journal of physiology* 290, H264-271.

Maines MD. (1997). The heme oxygenase system: a regulator of second messenger gases. *Annu Rev Pharmacol Toxicol* 37, 517-554.

Maines MD, Eke BC & Zhao X. (1996). Corticosterone promotes increased heme oxygenase-2 protein and transcript expression in the newborn rat brain. *Brain research* 722, 83-94.

Maines MD & Kappas A. (1974). Cobalt induction of hepatic heme oxygenase; with evidence that cytochrome P-450 is not essential for this enzyme activity. *Proceedings of the National Academy of Sciences of the United States of America* 71, 4293-4297.

Maines MD, Trakshel GM & Kutty RK. (1986). Characterization of two constitutive forms of rat liver microsomal heme oxygenase. Only one molecular species of the enzyme is inducible. *The Journal of biological chemistry* 261, 411-419.

Manukhina EB, Downey HF & Mallet RT. (2006). Role of nitric oxide in cardiovascular adaptation to intermittent hypoxia. *Experimental biology and medicine (Maywood, NJ* 231, 343-365.

Marks GS, Brien JF & Nakatsu K. (2003). What role does the heme-- heme oxygenase--carbon monoxide system play in vasoregulation? *American journal of physiology* 285, R522-523.

Marks GS, Brien JF, Nakatsu K & McLaughlin BE. (1991). Does carbon monoxide have a physiological function? *Trends in pharmacological sciences* 12, 185-188.

Marks GS, McLaughlin BE, Vreman HJ, Stevenson DK, Nakatsu K, Brien JF & Pang SC. (1997). Heme oxygenase activity and immunohistochemical localization in bovine pulmonary artery and vein. *Journal of cardiovascular pharmacology* 30, 1-6.

Marshall JJ & Kontos HA. (1990). Endothelium-derived relaxing factors. A perspective from in vivo data. *Hypertension* 16, 371-386.

Masic LP. (2006). Arginine mimetic structures in biologically active antagonists and inhibitors. *Curr Med Chem* 13(30), 3627-3648.

Matoba T, Shimokawa H, Nakashima M, Hirakawa Y, Mukai Y, Hirano K, Kanaide H & Takeshita A. (2000). Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *The Journal of clinical investigation* 106, 1521-1530.

Matsuoka A, Stuehr DJ, Olson JS, Clark P & Ikeda-Saito M. (1994). L-arginine and calmodulin regulation of the heme iron reactivity in neuronal nitric oxide synthase. *The Journal of biological chemistry* 269, 20335-20339.

May BK, Bhasker CR, Bawden MJ & Cox TC. (1990). Molecular regulation of 5-aminolevulinate synthase. Diseases related to heme biosynthesis. *Molecular biology & medicine* 7, 405-421.

McCarron JG, Osol G & Halpern W. (1989). Myogenic responses are independent of the endothelium in rat pressurized posterior cerebral arteries. *Blood vessels* 26, 315-319.

McCoubrey WK, Jr., Huang TJ & Maines MD. (1997). Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. *European journal of biochemistry / FEBS* 247, 725-732.

McMillan K, Bredt DS, Hirsch DJ, Snyder SH, Clark JE & Masters BS. (1992). Cloned, expressed rat cerebellar nitric oxide synthase contains stoichiometric amounts of heme, which binds carbon monoxide. *Proceedings of the National Academy of Sciences of the United States of America* 89, 11141-11145.

McSherry IN, Sandow SL, Campbell WB, Falck JR, Hill MA & Dora KA. (2006). A role for heterocellular coupling and EETs in dilation of rat cremaster arteries. *Microcirculation* 13, 119-130.

Meininger GA & Davis MJ. (1992). Cellular mechanisms involved in the vascular myogenic response. *The American journal of physiology* 263, H647-659.

Meininger GA, Mack CA, Fehr KL & Bohlen HG. (1987). Myogenic vasoregulation overrides local metabolic control in resting rat skeletal muscle. *Circulation research* 60, 861-870.

Meredith IT, Currie KE, Anderson TJ, Roddy MA, Ganz P & Creager MA. (1996). Postischemic vasodilation in human forearm is dependent on endothelium-derived nitric oxide. *The American journal of physiology* 270, H1435-1440.

Messina EJ, Weiner R & Kaley G. (1977). Arteriolar reactive hyperemia: modification by inhibitors of prostaglandin synthesis. *The American journal of physiology* 232, H571-575.

Miller JA, Velayo NL, Dage RC & Rampe D. (1991). High affinity [3H]glibenclamide binding sites in rat neuronal and cardiac tissue: localization and developmental characteristics. *The Journal of pharmacology and experimental therapeutics* 256, 358-364.

Miura H, Bosnjak JJ, Ning G, Saito T, Miura M & Gutterman DD. (2003). Role for hydrogen peroxide in flow-induced dilation of human coronary arterioles. *Circulation research* 92, e31-40.

Miura H, Liu Y & Gutterman DD. (1999). Human coronary arteriolar dilation to bradykinin depends on membrane hyperpolarization: contribution of nitric oxide and Ca^{2+}-activated K^{+} channels. *Circulation* 99, 3132-3138.

Moens AL, Goovaerts I, Claeys MJ & Vrints CJ. (2005). Flow-mediated vasodilation: a diagnostic instrument, or an experimental tool? *Chest* 127, 2254-2263.

Moncada S, Gryglewski R, Bunting S & Vane JR. (1976). An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature* 263, 663-665.

Moncada S & Higgs EA. (1995). Molecular mechanisms and therapeutic strategies related to nitric oxide. *Faseb J* 9, 1319-1330.

Moncada S, Palmer RM & Higgs EA. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacological reviews* 43, 109-142.

Morimoto Y, Durante W, Lancaster DG, Klattenhoff J & Tittel FK. (2001). Real-time measurements of endogenous CO production from vascular cells using an ultrasensitive laser sensor. *Am J Physiol Heart Circ Physiol* 280, H483-488.

Morita T, Perrella MA, Lee ME & Kourembanas S. (1995). Smooth muscle cell-derived carbon monoxide is a regulator of vascular cGMP. *Proceedings of the National Academy of Sciences of the United States of America* 92, 1475-1479.

Moritoki H, Iwamoto T, Kanaya J, Ishida Y & Fukuda H. (1986). Age-related change in serotonin-mediated prejunctional inhibition of rat vas deferens. *European journal of pharmacology* 132, 39-46.

Motterlini R, Foresti R, Intaglietta M & Winslow RM. (1996). NO-mediated activation of heme oxygenase: endogenous cytoprotection against oxidative stress to endothelium. *The American journal of physiology* 270, H107-114.

Motterlini R, Gonzales A, Foresti R, Clark JE, Green CJ & Winslow RM. (1998). Heme oxygenase-1-derived carbon monoxide contributes to the suppression of acute hypertensive responses in vivo. *Circulation research* 83, 568-577.

Mueggler PA, Peterson JS, Koler RD, Metcalfe J & Black JA. (1979). Postnatal regulation of oxygen delivery: hematologic parameters of postnatal dogs. *The American journal of physiology* 237, H71-75.

Mullarkey CJ, Edelstein D & Brownlee M. (1990). Free radical generation by early glycation products: a mechanism for accelerated atherogenesis in diabetes. *Biochemical and biophysical research communications* 173, 932-939.

Muller JM, Davis MJ, Kuo L & Chilian WM. (1999). Changes in coronary endothelial cell Ca^{2+} concentration during shear stress- and agonist-induced vasodilation. *The American journal of physiology* 276, H1706-1714.

Muller RM, Taguchi H & Shibahara S. (1987). Nucleotide sequence and organization of the rat heme oxygenase gene. *The Journal of biological chemistry* 262, 6795-6802.

Mulvany MJ & Halpern W. (1976). Mechanical properties of vascular smooth muscle cells in situ. *Nature* 260, 617-619.

Munzel T, Sayegh H, Freeman BA, Tarpey MM & Harrison DG. (1995). Evidence for enhanced vascular superoxide anion production in nitrate tolerance. A novel mechanism underlying tolerance and cross-tolerance. *The Journal of clinical investigation* 95, 187-194.

Musicki B & Burnett AL. (2006). eNOS function and dysfunction in the penis. *Experimental biology and medicine (Maywood, NJ* 231, 154-165.

Naik JS, O'Donaughy TL & Walker BR. (2003). Endogenous carbon monoxide is an endothelial-derived vasodilator factor in the mesenteric circulation. *Am J Physiol Heart Circ Physiol* 284, H838-845.

Nakazono K, Watanabe N, Matsuno K, Sasaki J, Sato T & Inoue M. (1991). Does superoxide underlie the pathogenesis of hypertension? *Proceedings of the National Academy of Sciences of the United States of America* 88, 10045-10048.

Narayanan J, Imig M, Roman RJ & Harder DR. (1994). Pressurization of isolated renal arteries increases inositol trisphosphate and diacylglycerol. *The American journal of physiology* 266, H1840-1845.

Nathan C. (1992). Nitric oxide as a secretory product of mammalian cells. *Faseb J* 6, 3051-3064.

Nelson MT, Cheng H, Rubart M, Santana LF, Bonev AD, Knot HJ & Lederer WJ. (1995). Relaxation of arterial smooth muscle by calcium sparks. *Science* 270, 633-637.

Nelson MT, Conway MA, Knot HJ & Brayden JE. (1997). Chloride channel blockers inhibit myogenic tone in rat cerebral arteries. *The Journal of physiology* 502 (Pt 2), 259-264.

Nelson MT, Patlak JB, Worley JF & Standen NB. (1990). Calcium channels, potassium channels, and voltage dependence of arterial smooth muscle tone. *The American journal of physiology* 259, C3-18.

Nelson MT & Quayle JM. (1995). Physiological roles and properties of potassium channels in arterial smooth muscle. *The American journal of physiology* 268, C799-822.

Neunteufl T, Heher S, Katzenschlager R et al. (2000). Late prognostic value of flow-mediated dilatation in the brachial artery of patients with chest pain. *Am J Cardiol* 86, 207-210.

Nguyen TD, Vequaud P & Thorin E. (1999). Effects of endothelin receptor antagonists and nitric oxide on myogenic tone and alpha-adrenergic-dependent contractions of rabbit resistance arteries. *Cardiovascular research* 43, 755-761.

North AJ, Lau KS, Brannon TS, Wu LC, Wells LB, German Z & Shaul PW. (1996). Oxygen upregulates nitric oxide synthase gene expression in ovine fetal pulmonary artery endothelial cells. *The American journal of physiology* 270, L643-649.

Nurkiewicz TR & Boegehold MA. (2004). Calcium-independent release of endothelial nitric oxide in the arteriolar network: onset during rapid juvenile growth. *Microcirculation* 11, 453-462.

O'Donnell DC, Tod ML & Gordon JB. (1996). Developmental changes in endothelium-dependent relaxation of pulmonary arteries: role of EDNO and prostanoids. *J Appl Physiol* 81, 2013-2019.

O'Leary DS, Dunlap RC & Glover KW. (1994). Role of endothelium-derived relaxing factor in hindlimb reactive and active hyperemia in conscious dogs. *The American journal of physiology* 266, R1213-1219.

Olesen SP, Munch E, Moldt P & Drejer J. (1994). Selective activation of Ca^{2+}-dependent K^{+} channels by novel benzimidazolone. *European journal of pharmacology* 251, 53-59.

Oskarsson HJ & Hofmeyer TG. (1997). Diabetic human platelets release a substance that inhibits platelet-mediated vasodilation. *The American journal of physiology* 273, H371-379.

Osol G. (1991). Myogenic properties of blood vessels in vitro. In *The Resistance Vasculature*, ed. Bevan JA. Humana Press.

Osol G & Halpern W. (1985). Myogenic properties of cerebral blood vessels from normotensive and hypertensive rats. *The American journal of physiology* 249, H914-921.

Osol G, Laher I & Cipolla M. (1991). Protein kinase C modulates basal myogenic tone in resistance arteries from the cerebral circulation. *Circulation research* 68, 359-367.

Otterbein LE & Choi AM. (2000). Heme oxygenase: colors of defense against cellular stress. *Am J Physiol Lung Cell Mol Physiol* 279, L1029-1037.

Pagano PJ, Clark JK, Cifuentes-Pagano ME, Clark SM, Callis GM & Quinn MT. (1997). Localization of a constitutively active, phagocyte-like NADPH oxidase in rabbit aortic adventitia: enhancement by angiotensin II. *Proceedings of the National Academy of Sciences of the United States of America* 94, 14483-14488.

Pagano PJ, Ito Y, Tornheim K, Gallop PM, Tauber AI & Cohen RA. (1995). An NADPH oxidase superoxide-generating system in the rabbit aorta. *The American journal of physiology* 268, H2274-2280.

Palmer RM, Ashton DS & Moncada S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* 333, 664-666.

Parfenova H, Massie V & Leffler CW. (2000). Developmental changes in endothelium-derived vasorelaxant factors in cerebral circulation. *American journal of physiology* 278, H780-788.

Parfenova H, Neff RA, 3rd, Alonso JS, Shlopov BV, Jamal CN, Sarkisova SA & Leffler CW. (2001). Cerebral vascular endothelial heme oxygenase: expression, localization, and activation by glutamate. *Am J Physiol Cell Physiol* 281, C1954-1963.

Park JB, Charbonneau F, Schiffrin EL. (2001). Correlation of endothelial function in large and small arteries in human essential hypertension. *J Hypertens* 19, 415-420.

Pearce WJ & Elliott SR. (1994). Maturation enhances the sensitivity of ovine cerebral arteries to the ATP-sensitive potassium channel activator lemakalim. *Pediatric research* 35, 729-732.

Pearce WJ, Hull AD, Long DM & White CR. (1994). Effects of maturation on cyclic GMP-dependent vasodilation in ovine basilar and carotid arteries. *Pediatric research* 36, 25-33.

Pelaez NJ, Braun TR, Paul RJ, Meiss RA & Packer CS. (2000a). H_2O_2 mediates Ca^{2+}- and MLC(20) phosphorylation-independent contraction in intact and permeabilized vascular muscle. *American journal of physiology* 279, H1185-1193.

Pelaez NJ, Osterhaus SL, Mak AS, Zhao Y, Davis HW & Packer CS. (2000b). MAPK and PKC activity are not required for H_2O_2 -induced arterial muscle contraction. *American journal of physiology* 279, H1194-1200.

Penney DG. (1990). Acute carbon monoxide poisoning: animal models: a review. *Toxicology* 62, 123-160.

Perrella MA & Yet SF. (2003). Role of heme oxygenase-1 in cardiovascular function. *Current pharmaceutical design* 9, 2479-2487.

Pick E & Keisari Y. (1980). A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. *Journal of immunological methods* 38, 161-170.

Plut C, Ribiere C, Giudicelli Y & Dausse JP. (2002). Gender differences in hypothalamic tyrosine hydroxylase and alpha(2)-adrenoceptor subtype gene expression in cafeteria diet-induced hypertension and consequences of neonatal androgenization. *The Journal of pharmacology and experimental therapeutics* 302, 525-531.

Pohl U & Busse R. (1989). Hypoxia stimulates release of endothelium-derived relaxant factor. *The American journal of physiology* 256, H1595-1600.

Pohl U & Busse R. (1990). Endothelium-dependent modulation of vascular tone and platelet function. *European heart journal* 11 Suppl B, 35-42.

Prewitt RL, Chen, II & Dowell R. (1982). Development of microvascular rarefaction in the spontaneously hypertensive rat. *The American journal of physiology* 243, H243-251.

Pries AR & Kuebler WM. (2006). Normal endothelium. *Handbook of experimental pharmacology*, 1-40.

Prior HM, Yates MS & Beech DJ. (1998). Functions of large conductance Ca^{2+}-activated (BK_{Ca}), delayed rectifier (K_V) and background K^+ channels in the control of membrane potential in rabbit renal arcuate artery. *The Journal of physiology* 511 (Pt 1), 159-169.

Proctor KG, Damon DN & Duling BR. (1981). Tissue PO_2 and arteriolar responses to metabolic stimuli during maturation of striated muscle. *The American journal of physiology* 241, H325-331.

Pryor WA, Houk KN, Foote CS, Fukuto JM, Ignarro LJ, Squadrito GL & Davies KJ. (2006). Free radical biology and medicine: it's a gas, man! *Am J Physiol Regul Integr Comp Physiol* 291, R491-511.

Pufahl RA & Marletta MA. (1993). Oxidation of NG-hydroxy-L-arginine by nitric oxide synthase: evidence for the involvement of the heme in catalysis. *Biochemical and biophysical research communications* 193, 963-970.

Quayle JM, McCarron JG, Brayden JE & Nelson MT. (1993). Inward rectifier K^+ currents in smooth muscle cells from rat resistance-sized cerebral arteries. *The American journal of physiology* 265, C1363-1370.

Quayle JM, Nelson MT & Standen NB. (1997). ATP-sensitive and inwardly rectifying potassium channels in smooth muscle. *Physiol Rev* 77, 1165-1232.

Rajagopalan S, Kurz S, Munzel T, Tarpey M, Freeman BA, Griendling KK & Harrison DG. (1996). Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *The Journal of clinical investigation* 97, 1916-1923.

Rees DD, Palmer RM & Moncada S. (1989). Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proceedings of the National Academy of Sciences of the United States of America* 86, 3375-3378.

Reeve HL, Weir EK, Archer SL & Cornfield DN. (1998). A maturational shift in pulmonary K^+ channels, from Ca^{2+} sensitive to voltage dependent. *The American journal of physiology* 275, L1019-1025.

Rees DD, Palmer RM, Schulz R, Hodson HF, Moncada S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. Br J Pharmacol 101(3), 746-752.

Reid MB. (1998). Role of nitric oxide in skeletal muscle: synthesis, distribution and functional importance. *Acta physiologica Scandinavica* 162, 401-409.

Resnick N, Yahav H, Shay-Salit A, Shushy M, Schubert S, Zilberman LC & Wofovitz E. (2003). Fluid shear stress and the vascular endothelium: for better and for worse. *Progress in biophysics and molecular biology* 81, 177-199.

Ricci F & Palleschi G. (2005). Sensor and biosensor preparation, optimisation and applications of Prussian Blue modified electrodes. *Biosensors & bioelectronics* 21, 389-407.

Ripoll E, Sillau AH & Banchero N. (1979). Changes in the capillarity of skeletal muscle in the growing rat. *Pflugers Arch* 380, 153-158.

Risau W. (1995). Differentiation of endothelium. *Faseb J* 9, 926-933.

Roberts GP, Youn H & Kerby RL. (2004). CO-sensing mechanisms. *Microbiol Mol Biol Rev* 68, 453-473.

Rodgers PA, Vreman HJ, Dennery PA & Stevenson DK. (1994). Sources of carbon monoxide (CO) in biological systems and applications of CO detection technologies. *Seminars in perinatology* 18, 2-10.

Rodriguez-Martinez MA, Garcia-Cohen EC, Baena AB, Gonzalez R, Salaices M & Marin J. (1998). Contractile responses elicited by hydrogen peroxide in aorta from normotensive and hypertensive rats. Endothelial modulation and mechanism involved. *British journal of pharmacology* 125, 1329-1335.

Roman RJ, Maier KG, Sun CW, Harder DR & Alonso-Galicia M. (2000). Renal and cardiovascular actions of 20-hydroxyeicosatetraenoic acid and epoxyeicosatrienoic acids. *Clinical and experimental pharmacology & physiology* 27, 855-865.

Rongen GA, Smits P & Thien T. (1994). Endothelium and the regulation of vascular tone with emphasis on the role of nitric oxide. Physiology, pathophysiology and clinical implications. *The Netherlands journal of medicine* 44, 26-35.

Rubanyi GM. (1988). Endothelium-dependent pressure-induced contraction of isolated canine carotid arteries. *The American journal of physiology* 255, H783-788.

Rubanyi GM. (1993). The role of endothelium in cardiovascular homeostasis and diseases. *Journal of cardiovascular pharmacology* 22 Suppl 4, S1-14.

Rubanyi GM, Romero JC & Vanhoutte PM. (1986). Flow-induced release of endothelium-derived relaxing factor. *The American journal of physiology* 250, H1145-1149.

Ruch W, Cooper PH & Baggiolini M. (1983). Assay of H_2O_2 production by macrophages and neutrophils with homovanillic acid and horse-radish peroxidase. *Journal of immunological methods* 63, 347-357.

Rueben A, Popp R, Busse R, & Fleming I. (2004). Cytochrome P450 2c-derived epoxyeicosatrienoic acids (EETs) regulate Ca^{2+} signaling and hyperpolarization in endothelial cells. *J Vasc Res* 41, 460.

Ryter SW & Otterbein LE. (2004). Carbon monoxide in biology and medicine. *Bioessays* 26, 270-280.

Ryter SW, Otterbein LE, Morse D & Choi AM. (2002). Heme oxygenase/carbon monoxide signaling pathways: regulation and functional significance. *Molecular and cellular biochemistry* 234-235, 249-263.

Ryter SW & Tyrrell RM. (2000). The heme synthesis and degradation pathways: role in oxidant sensitivity. Heme oxygenase has both pro- and antioxidant properties. *Free radical biology & medicine* 28, 289-309.

Sacerdoti D, Escalante B, Abraham NG, McGiff JC, Levere RD & Schwartzman ML. (1989). Treatment with tin prevents the development of hypertension in spontaneously hypertensive rats. *Science* 243, 388-390.

Sachs F & Morris CE. (1998). Mechanosensitive ion channels in nonspecialized cells. *Reviews of physiology, biochemistry and pharmacology* 132, 1-77.

Sammut IA, Foresti R, Clark JE, Exon DJ, Vesely MJ, Sarathchandra P, Green CJ & Motterlini R. (1998). Carbon monoxide is a major contributor to the regulation of vascular tone in aortas expressing high levels of haeme oxygenase-1. *British journal of pharmacology* 125, 1437-1444.

Sandow SL, Tare M, Coleman HA, Hill CE & Parkington HC. (2002). Involvement of myoendothelial gap junctions in the actions of endothelium-derived hyperpolarizing factor. *Circulation research* 90, 1108-1113.

Sarelius IH, Damon DN & Duling BR. (1981). Microvascular adaptations during maturation of striated muscle. *The American journal of physiology* 241, H317-324.

Schmidt HH. (1992). NO., CO and .OH. Endogenous soluble guanylyl cyclase-activating factors. *FEBS letters* 307, 102-107.

Segal SS. (2000). Integration of blood flow control to skeletal muscle: key role of feed arteries. *Acta physiologica Scandinavica* 168, 511-518.

Segal SS. (2005). Regulation of blood flow in the microcirculation. *Microcirculation* 12, 33-45.

Sener A & Smith FG. (2001). Renal hemodynamic effects of L-NAME during postnatal maturation in conscious lambs. *Pediatric nephrology (Berlin, Germany)* 16, 868-873.

Sessa WC, Harrison JK, Barber CM, Zeng D, Durieux ME, D'Angelo DD, Lynch KR & Peach MJ. (1992). Molecular cloning and expression of a cDNA encoding endothelial cell nitric oxide synthase. *The Journal of biological chemistry* 267, 15274-15276.

Sharp PE & La Regina MC. (1998). *The Laboratory Rat*. CRC Press, Boca Raton.

Shepherd JT. (1964). Reactive Hyperemia in Human Extremities. *Circulation research* 15, SUPPL:76-79.

Sherwood L. (2007). *Human Physiology: From Cells to Systems*. Thomson Brooks/Cole, United States.

Shimizu S, Shiota K, Yamamoto S, Miyasaka Y, Ishii M, Watabe T, Nishida M, Mori Y, Yamamoto T & Kiuchi Y. (2003). Hydrogen peroxide stimulates tetrahydrobiopterin synthesis through the induction of GTP-cyclohydrolase I and increases nitric oxide synthase activity in vascular endothelial cells. *Free radical biology & medicine* 34, 1343-1352.

Shiojima I & Walsh K. (2002). Role of Akt signaling in vascular homeostasis and angiogenesis. *Circulation research* 90, 1243-1250.

Shiotsuka RN, Drew RT & Wehner RW. (1984). Carbon monoxide enhances development of hypertension in Dahl rats. *Toxicology and applied pharmacology* 76, 225-233.

Sillau AH & Banchero N. (1977). Effect of maturation on capillary density, fiber size and composition in rat skeletal muscle. *Proceedings of the Society for Experimental*

Biology and Medicine Society for Experimental Biology and Medicine (New York, NY 154, 461-466.

Silveira LR, Pereira-Da-Silva L, Juel C & Hellsten Y. (2003). Formation of hydrogen peroxide and nitric oxide in rat skeletal muscle cells during contractions. *Free radical biology & medicine* 35, 455-464.

Sjostrand T. (1949). Endogenous formation of carbon monoxide in man under normal and pathological conditions. *Scan J Clin Lab Invest* 1, 201–214.

Smith KR, Klei LR & Barchowsky A. (2001). Arsenite stimulates plasma membrane NADPH oxidase in vascular endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 280, L442-449.

Sobey CG, Heistad DD & Faraci FM. (1997). Mechanisms of bradykinin-induced cerebral vasodilatation in rats. Evidence that reactive oxygen species activate K^+ channels. *Stroke; a journal of cerebral circulation* 28, 2290-2294; discussion 2295.

Sohn HY, Krotz F, Gloe T, Keller M, Theisen K, Klauss V & Pohl U. (2003). Differential regulation of xanthine and NAD(P)H oxidase by hypoxia in human umbilical vein endothelial cells. Role of nitric oxide and adenosine. *Cardiovascular research* 58, 638-646.

Solhaug MJ, Dong XQ, Adelman RD & Dong KW. (2000). Ontogeny of neuronal nitric oxide synthase, NOS I, in the developing porcine kidney. *Am J Physiol Regul Integr Comp Physiol* 278, R1453-1459.

Sotnikova R. (1998). Investigation of the mechanisms underlying H_2O_2 -evoked contraction in the isolated rat aorta. *General pharmacology* 31, 115-119.

Standen NB, Quayle JM, Davies NW, Brayden JE, Huang Y & Nelson MT. (1989). Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science* 245, 177-180.

Staniek K & Nohl H. (1999). H_2O_2 detection from intact mitochondria as a measure for one-electron reduction of dioxygen requires a non-invasive assay system. *Biochimica et biophysica acta* 1413, 70-80.

Steinhorn RH, Morin FC, 3rd & Russell JA. (1994). The adventitia may be a barrier specific to nitric oxide in rabbit pulmonary artery. *The Journal of clinical investigation* 94, 1883-1888.

Stoen R, Brubakk AM, Vik T, Lossius K, Jynge P & Karlsson JO. (1997). Postnatal changes in mechanisms mediating acetylcholine-induced relaxation in piglet femoral arteries. *Pediatric research* 41, 702-707.

Stone JR & Marletta MA. (1994). Soluble guanylate cyclase from bovine lung: activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states. *Biochemistry* 33, 5636-5640.

Strobaek D, Jorgensen TD, Christophersen P, Ahring PK & Olesen SP. (2000). Pharmacological characterization of small-conductance Ca^{2+}-activated K^{+} channels stably expressed in HEK 293 cells. *British journal of pharmacology* 129, 991-999.

Su BY, Reber KM, Nankervis CA & Nowicki PT. (2003). Development of the myogenic response in postnatal intestine: role of PKC. *Am J Physiol Gastrointest Liver Physiol* 284, G445-452.

Suttorp N, Toepfer W & Roka L. (1986). Antioxidant defense mechanisms of endothelial cells: glutathione redox cycle versus catalase. *The American journal of physiology* 251, C671-680.

Syme CA, Gerlach AC, Singh AK & Devor DC. (2000). Pharmacological activation of cloned intermediate- and small-conductance Ca^{2+}-activated K^{+} channels. *Am J Physiol Cell Physiol* 278, C570-581.

Szasz T, Thakali K, Fink GD & Watts SW. (2007). A comparison of arteries and veins in oxidative stress: producers, destroyers, function, and disease. *Experimental biology and medicine (Maywood, NJ* 232, 27-37.

Tabet F, Savoia C, Schiffrin EL & Touyz RM. (2004). Differential calcium regulation by hydrogen peroxide and superoxide in vascular smooth muscle cells from spontaneously hypertensive rats. *Journal of cardiovascular pharmacology* 44, 200-208.

Tanaka Y, Koike K & Toro L. (2004). MaxiK channel roles in blood vessel relaxations induced by endothelium-derived relaxing factors and their molecular mechanisms. *Journal of smooth muscle research = Nihon Heikatsukin Gakkai kikanshi* 40, 125-153.

Tao J, Yang Z, Wang JM, Wang LC, Luo CF, Tang AL, Dong YG & Ma H. (2007). Shear stress increases Cu/Zn SOD activity and mRNA expression in human endothelial progenitor cells. *Journal of human hypertension* 21, 353-358.

Teitel D & Rudolph AM. (1985). Perinatal oxygen delivery and cardiac function. *Advances in pediatrics* 32, 321-347.

Teng GQ, Nauli SM, Brayden JE & Pearce WJ. (2002). Maturation alters the contribution of potassium channels to resting and 5HT-induced tone in small cerebral arteries of the sheep. *Brain research* 133, 81-91.

Test ST & Weiss SJ. (1984). Quantitative and temporal characterization of the extracellular H_2O_2 pool generated by human neutrophils. *The Journal of biological chemistry* 259, 399-405.

Thengchaisri N & Kuo L. (2003). Hydrogen peroxide induces endothelium-dependent and -independent coronary arteriolar dilation: role of cyclooxygenase and potassium channels. *American journal of physiology* 285, H2255-2263.

Thom SR, Xu YA & Ischiropoulos H. (1997). Vascular endothelial cells generate peroxynitrite in response to carbon monoxide exposure. *Chemical research in toxicology* 10, 1023-1031.

Thompson LP & Weiner CP. (1996). Acetylcholine relaxation of renal artery and nitric oxide synthase activity of renal cortex increase with fetal and postnatal age. *Pediatric research* 40, 192-197.

Thomsen K, Rubin I & Lauritzen M. (2002). NO- and non-NO-, non-prostanoid-dependent vasodilatation in rat sciatic nerve during maturation and developing experimental diabetic neuropathy. *The Journal of physiology* 543, 977-993.

Thorup C, Jones CL, Gross SS, Moore LC & Goligorsky MS. (1999). Carbon monoxide induces vasodilation and nitric oxide release but suppresses endothelial NOS. *Am J Physiol* 277, F882-889.

Toma BS, Wangler RD, DeWitt DF & Sparks HV, Jr. (1985). Effect of development on coronary vasodilator reserve in the isolated guinea pig heart. *Circulation research* 57, 538-544.

Toth A, Pal M, Intaglietta M & Johnson PC. (2007). Contribution of anaerobic metabolism to reactive hyperemia in skeletal muscle. *American journal of physiology* 292, H2643-2653.

Trakshel GM, Kutty RK & Maines MD. (1986). Purification and characterization of the major constitutive form of testicular heme oxygenase. The noninducible isoform. *The Journal of biological chemistry* 261, 11131-11137.

Uluoglu C & Zengil H. (2003). Comparison of different de-endothelization procedures in the isolated rat thoracic aorta: a short communication. *Research communications in molecular pathology and pharmacology* 113-114, 289-297.

Ungvari Z, Csiszar A & Koller A. (2002). Increases in endothelial Ca^{2+} activate K_{Ca} channels and elicit EDHF-type arteriolar dilation via gap junctions. *American journal of physiology* 282, H1760-1767.

Ungvari Z, Sun D, Huang A, Kaley G & Koller A. (2001). Role of endothelial [Ca^{2+}]i in activation of eNOS in pressurized arterioles by agonists and wall shear stress. *American journal of physiology* 281, H606-612.

Unthank JL & Bohlen HG. (1988). Intestinal microvascular growth during maturation in diabetic juvenile rats. *Circulation research* 63, 429-436.

Ursini F, Maiorino M, Brigelius-Flohe R, Aumann KD, Roveri A, Schomburg D & Flohe L. (1995). Diversity of glutathione peroxidases. *Methods in enzymology* 252, 38-53.

van der Vliet A, Hu ML, O'Neill CA, Cross CE & Halliwell B. (1994). Interactions of human blood plasma with hydrogen peroxide and hypochlorous acid. *The Journal of laboratory and clinical medicine* 124, 701-707.

Vanhoutte PM. (1989). Endothelium and control of vascular function. State of the Art lecture. *Hypertension* 13, 658-667.

Vanhoutte PM & Scott-Burden T. (1994). The endothelium in health and disease. *Texas Heart Institute journal / from the Texas Heart Institute of St* 21, 62-67.

Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS, Karoui H, Tordo P & Pritchard KA, Jr. (1998). Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proceedings of the National Academy of Sciences of the United States of America* 95, 9220-9225.

Villar IC, Francis S, Webb A, Hobbs AJ & Ahluwalia A. (2006). Novel aspects of endothelium-dependent regulation of vascular tone. *Kidney international* 70, 840-853.

Vreeke MS, Maidan R & Heller A. (1994). Diagnostic biosensor polymers. In *ACS Symposium Series 556*, ed. Usmani AM & Akmal N, pp. 180-192. American Chemical Society, Washington D.C.

Vreman HJ, Ekstrand BC & Stevenson DK. (1993). Selection of metalloporphyrin heme oxygenase inhibitors based on potency and photoreactivity. *Pediatric research* 33, 195-200.

Vreman HJ, Wong RJ & Stevenson DK. (2000). Carbon monoxide in breath, blood, and other tissues. In *Carbon Monoxide Toxicity*, ed. Penney DG, pp. 19-50. CRC, Boca Raton, FL.

Wagner CT, Durante W, Christodoulides N, Hellums JD & Schafer AI. (1997). Hemodynamic forces induce the expression of heme oxygenase in cultured vascular smooth muscle cells. *The Journal of clinical investigation* 100, 589-596.

Waldman SA & Murad F. (1988). Biochemical mechanisms underlying vascular smooth muscle relaxation: the guanylate cyclase-cyclic GMP system. *Journal of cardiovascular pharmacology* 12 Suppl 5, S115-118.

Wallis SJ, Firth J & Dunn WR. (1996). Pressure-induced myogenic responses in human isolated cerebral resistance arteries. *Stroke; a journal of cerebral circulation* 27, 2287-2290; discussion 2291.

Wang DH & Prewitt RL. (1991). Microvascular development during normal growth and reduced blood flow: introduction of a new model. *The American journal of physiology* 260, H1966-1972.

Wang MH, Brand-Schieber E, Zand BA, Nguyen X, Falck JR, Balu N & Schwartzman ML. (1998). Cytochrome P450-derived arachidonic acid metabolism in the rat kidney: characterization of selective inhibitors. *The Journal of pharmacology and experimental therapeutics* 284, 966-973.

Wang R. (1998). Resurgence of carbon monoxide: an endogenous gaseous vasorelaxing factor. *Canadian journal of physiology and pharmacology* 76, 1-15.

Wang R, Wang Z & Wu L. (1997a). Carbon monoxide-induced vasorelaxation and the underlying mechanisms. *British journal of pharmacology* 121, 927-934.

Wang R & Wu L. (1997). The chemical modification of K_{Ca} channels by carbon monoxide in vascular smooth muscle cells. *The Journal of biological chemistry* 272, 8222-8226.

Wang R, Wu L & Wang Z. (1997b). The direct effect of carbon monoxide on K_{Ca} channels in vascular smooth muscle cells. *Pflugers Arch* 434, 285-291.

Wassmann S, Wassmann K & Nickenig G. (2004). Modulation of oxidant and antioxidant enzyme expression and function in vascular cells. *Hypertension* 44, 381-386.

Watson PA. (1991). Function follows form: generation of intracellular signals by cell deformation. *Faseb J* 5, 2013-2019.

Weaver LK. (1999). Carbon monoxide poisoning. *Critical care clinics* 15, 297-317, viii.

Wei EP, Kontos HA & Beckman JS. (1996). Mechanisms of cerebral vasodilation by superoxide, hydrogen peroxide, and peroxynitrite. *The American journal of physiology* 271, H1262-1266.

Welsh DG & Segal SS. (2000). Role of EDHF in conduction of vasodilation along hamster cheek pouch arterioles in vivo. *American journal of physiology* 278, H1832-1839.

Wesselman JP, Schubert R, VanBavel ED, Nilsson H & Mulvany MJ. (1997). K_{Ca}-channel blockade prevents sustained pressure-induced depolarization in rat mesenteric small arteries. *The American journal of physiology* 272, H2241-2249.

Wesselman JP, Spaan JA, van der Meulen ET & VanBavel E. (2001). Role of protein kinase C in myogenic calcium-contraction coupling of rat cannulated mesenteric small arteries. *Clinical and experimental pharmacology & physiology* 28, 848-855.

White CR, Hamade MW, Siami K, Chang MM, Mangalwadi A, Frangos JA & Pearce WJ. (2005). Maturation enhances fluid shear-induced activation of eNOS in perfused ovine carotid arteries. *American journal of physiology* 289, H2220-2227.

White KA & Marletta MA. (1992). Nitric oxide synthase is a cytochrome P-450 type hemoprotein. *Biochemistry* 31, 6627-6631.

Willis AP & Leffler CW. (1999). NO and prostanoids: age dependence of hypercapniaand histamine-induced dilations of pig pial arterioles. *The American journal of physiology* 277, H299-307.

Willis AP & Leffler CW. (2001). Endothelial NO and prostanoid involvement in newborn and juvenile pig pial arteriolar vasomotor responses. *American journal of physiology* 281, H2366-2377.

Willis D, Moore AR, Frederick R & Willoughby DA. (1996). Heme oxygenase: a novel target for the modulation of the inflammatory response. *Nature medicine* 2, 87-90.

Willis D, Tomlinson A, Frederick R, Paul-Clark MJ & Willoughby DA. (1995). Modulation of heme oxygenase activity in rat brain and spleen by inhibitors and donors of nitric oxide. *Biochemical and biophysical research communications* 214, 1152-1156.

Wilson LE, Levy M, Stuart-Smith K & Haworth SG. (1993). Postnatal adrenoreceptor maturation in porcine intrapulmonary arteries. *Pediatric research* 34, 591-595.

Wolin MS, Messina EJ & Kaley G. (1989). Involvement of prostaglandins in arteriolar vasodilation to peroxides. *Advances in prostaglandin, thromboxane, and leukotriene research* 19, 281-284.

Wolin MS, Rodenburg JM, Messina EJ & Kaley G. (1990). Similarities in the pharmacological modulation of reactive hyperemia and vasodilation to hydrogen peroxide in rat skeletal muscle arterioles: effects of probes for endothelium-derived mediators. *The Journal of pharmacology and experimental therapeutics* 253, 508-512.

Wu Y, Huang A, Sun D, Falck JR, Koller A & Kaley G. (2001). Gender-specific compensation for the lack of NO in the mediation of flow-induced arteriolar dilation. *American journal of physiology* 280, H2456-2461.

Wyatt AW, Steinert JR & Mann GE. (2004). Modulation of the L-arginine/nitric oxide signalling pathway in vascular endothelial cells. *Biochemical Society symposium*, 143-156.

Wyatt SB, Winters KP, & Dubbert PM. (2006). Overweight and obesity: prevalence, consequences, and causes of a growing public health problem. *Am J Med Sci*, 331(4), 166-174.

Xi Q, Tcheranova D, Parfenova H, Horowitz B, Leffler CW & Jaggar JH. (2004). Carbon monoxide activates K_{Ca} channels in newborn arteriole smooth muscle cells by increasing apparent Ca^{2+} sensitivity of alpha-subunits. *Am J Physiol Heart Circ Physiol* 286, H610-618.

Xia Y, Tsai AL, Berka V & Zweier JL. (1998). Superoxide generation from endothelial nitric-oxide synthase. A Ca^{2+}/calmodulin-dependent and tetrahydrobiopterin regulatory process. *The Journal of biological chemistry* 273, 25804-25808.

Yada T, Shimokawa H, Hiramatsu O, Kajita T, Shigeto F, Goto M, Ogasawara Y & Kajiya F. (2003). Hydrogen peroxide, an endogenous endothelium-derived hyperpolarizing factor, plays an important role in coronary autoregulation in vivo. *Circulation* 107, 1040-1045.

Yamamoto Y, Fukuta H, Nakahira Y & Suzuki H. (1998). Blockade by 18beta-glycyrrhetinic acid of intercellular electrical coupling in guinea-pig arterioles. *The Journal of physiology* 511 (Pt 2), 501-508.

Yamawaki H, Haendeler J & Berk BC. (2003). Thioredoxin: a key regulator of cardiovascular homeostasis. *Circulation research* 93, 1029-1033.

Yamazaki J, Duan D, Janiak R, Kuenzli K, Horowitz B & Hume JR. (1998). Functional and molecular expression of volume-regulated chloride channels in canine vascular smooth muscle cells. *The Journal of physiology* 507 (Pt 3), 729-736.

Yang ZW, Zhang A, Altura BT & Altura BM. (1998a). Endothelium-dependent relaxation to hydrogen peroxide in canine basilar artery: a potential new cerebral dilator mechanism. *Brain research bulletin* 47, 257-263.

Yang ZW, Zheng T, Wang J, Zhang A, Altura BT & Altura BM. (1999). Hydrogen peroxide induces contraction and raises $[Ca^{2+}]i$ in canine cerebral arterial smooth muscle: participation of cellular signaling pathways. *Naunyn-Schmiedeberg's archives of pharmacology* 360, 646-653.

Yang ZW, Zheng T, Zhang A, Altura BT & Altura BM. (1998b). Mechanisms of hydrogen peroxide-induced contraction of rat aorta. *European journal of pharmacology* 344, 169-181.

Yet SF, Pellacani A, Patterson C, Tan L, Folta SC, Foster L, Lee WS, Hsieh CM & Perrella MA. (1997). Induction of heme oxygenase-1 expression in vascular smooth muscle cells. A link to endotoxic shock. *The Journal of biological chemistry* 272, 4295-4301.

Yoshida T & Kikuchi G. (1978). Features of the reaction of heme degradation catalyzed by the reconstituted microsomal heme oxygenase system. *The Journal of biological chemistry* 253, 4230-4236.

Yoshida T, Takahashi S & Kikuchi G. (1974). Partial purification and reconstitution of the heme oxygenase system from pig spleen microsomes. *Journal of biochemistry* 75, 1187-1191.

Zatta AJ & Headrick JP. (2005). Mediators of coronary reactive hyperaemia in isolated mouse heart. *British journal of pharmacology* 144, 576-587.

Zeiher AM, Drexler H, Wollschlager H, & Just H. (1991). Modulation of coronary vasomotor tone in humans. Progressive endothelial dysfunction with different early stages of coronary atherosclerosis. *Circulation* 83, 391-401.

Zellers TM & Vanhoutte PM. (1991). Endothelium-dependent relaxations of piglet pulmonary arteries augment with maturation. *Pediatric research* 30, 176-180.

Zembowicz A, Hatchett RJ, Jakubowski AM & Gryglewski RJ. (1993). Involvement of nitric oxide in the endothelium-dependent relaxation induced by hydrogen peroxide in the rabbit aorta. *British journal of pharmacology* 110, 151-158.

Zhang F, Kaide J, Wei Y, Jiang H, Yu C, Balazy M, Abraham NG, Wang W & Nasjletti A. (2001a). Carbon monoxide produced by isolated arterioles attenuates pressure-induced vasoconstriction. *Am J Physiol Heart Circ Physiol* 281, H350-358.

Zhang Y, Oltman CL, Lu T, Lee HC, Dellsperger KC & VanRollins M. (2001b). EET homologs potently dilate coronary microvessels and activate BK(Ca) channels. *American journal of physiology* 280, H2430-2440.

Zitzmann M, Brune M & Nieschlag E. (2002). Vascular reactivity in hypogonadal men is reduced by androgen substitution. *The Journal of clinical endocrinology and metabolism* 87, 5030-5037.

Zou AP, Fleming JT, Falck JR, Jacobs ER, Gebremedhin D, Harder DR & Roman RJ. (1996). 20-HETE is an endogenous inhibitor of the large-conductance Ca^{2+}-

activated K^+ channel in renal arterioles. *The American journal of physiology* 270, R228-237.

Zou H, Ratz PH & Hill MA. (2000). Temporal aspects of Ca^{2+} and myosin phosphorylation during myogenic and norepinephrine-induced arteriolar constriction. *Journal of vascular research* 37, 556-567.

Zuckerman SL, Armstead WM, Hsu P, Shibata M & Leffler CW. (1996). Age dependence of cerebrovascular response mechanisms in domestic pigs. *The American journal of physiology* 271, H535-540.

Zweifach BW, Kovalcheck S, De Lano F & Chen P. (1981). Micropressure-flow relationships in a skeletal muscle of spontaneously hypertensive rats. *Hypertension* 3, 601-614.

IX. Techniques/programs/software training during PhD experience

AcqKnowledge Software

DataTrax

EndNote X

Kodak 1D 3.6

Metamorph 6.01

Microsoft Office Suite

Optimus 6.2

Sigma Stat 3.0

Sigma Plot 9.0

Catheterization of the carotid artery and jugular vein

Isolated Vessel System

Cell Culture (human alveolar cell line A549; rat alveolar macrophages)

Olympus IX70 inverted microscope (with Dage CCD-72S monochrome camera) to determine % cellular confluence

Pressure-flow BET instrument to determine surface area of particles

JEOL 6400 Scanning Electron Microscope to determine particulate diameter

Intratracheal instillation

Bronchoalveolar lavage (BAL)

Spinotrapezius preparation (for *in vivo* microscopy)

Iontophoresis

Intraluminal Injection

Myobath vessel system

Western Blot

Skin-fold test

Catheterization of the femoral artery and vein

Hind limb preparation (isolation of soleus, gastrocnemius, and plantaris) for force transduction analysis after electrical stimulation and vascular flow and resistance measurements (femoral artery)

Recovery surgery (sterile technique)

Orchidectomy

Venous and Arterial anastomosis

End-to-end and end-to-side technique

Right-side-up technique

Vein grafting

Renal transplants

Proficiency obtained with fine forceps, needle holders, vannas, vascular clamps, clamp applicators, and vessel dilators

X. CURRICULUM VITAE

Name:	Julie Balch Samora
Internet:	jbsamora@hsc.wvu.edu
Home Address:	1367 Headlee Ave Morgantown, West Virginia 26505
Home Phone:	(304) 598-3603
Cell Phone:	(304) 685-3062
Birth Date; Location:	May 26, 1977; Morgantown, WV
Marital Status:	Married
Number of Dependents:	Two
Name of Spouse:	Walter Paul Samora, III ("Quincy")
Name of Dependents:	Ethan Christopher Samora (2.5 years) Erin Michelle Samora (9 months)
Citizenship:	United States of America

Education:

2009	M.D. candidate, West Virginia University School of Medicine; Morgantown, WV
2007	Ph.D. candidate in Cellular & Integrative Physiology, West Virginia University School of Medicine, *Advisors: Matthew A. Boegehold, Ph.D. & Jefferson C. Frisbee, Ph.D.*
2005	M.P.A. with a specialization in Healthcare Administration, West Virginia University
2004	M.P.H., West Virginia University; Morgantown, WV
2001	M.M. in Bassoon Performance, Yale University; New Haven, CT
1999	B.F.A. in Music Performance with an additional major in Biological Sciences, Carnegie Mellon University; Pittsburgh, PA
1995	Interlochen Arts Academy; Interlochen, MI

Continuing Education:

2006	Christine M. Kleinert Institute Microsurgery Teaching Laboratory; Louisville, KT; Received training in microscopic vascular anastomosis (both arterial and venous); proficiency obtained with fine forceps, needle holders, vannas, vascular clamps, clamp applicators, and vessel dilators; end-to-end as well as end-to-side anastomosis; interpositional grafting; right-side-up technique; use of foot switch controls and motorized zoom/focus
2006	Certificate in Women's Health
2005	Certificate in Healthcare Administration
2004	Community Health Education Specialist (CHES)

Professional Society Memberships:

Phi Beta Kappa
Pi Alpha Alpha (The National Honor Society for Public Affairs and Administration)
Pi Kappa Lambda (Music Honor Society)
Sigma Xi (The Scientific Research Society)
Microcirculatory Society, USA, student member
Society for Experimental Biology and Medicine, student member
American College of Surgeons, student member
American College of Preventive Medicine, Medical Student Section
American College of Physicians, student member
American Medical Association, Medical Student Section
American Medical Student Association
American Alliance for Health, Physical Education, Recreation and Dance (AAHPERD)
American Association for Health Education
West Virginia State Medical Association, student member

Institutional Service:

2006-2008	Institutional Review Board
2005-2008	Curriculum Committee, WVU School of Medicine
2005-2007	American Physician Scientists Association (APSA) Institutional Representative for West Virginia University
2005-2006	APSA Public Policy Committee
2003-2004	Search Committee Member for the Dean, WVU School of Medicine
1996-1997	Carnegie Mellon University "Ambassador"

Leadership Roles/Extracurricular Activities:

2007-2008	Northern WV Rural Health Education Center (NWVRHEC) Board Member
2003-2004	Leadership Council, Community Medicine Student Association
2002-2003	President, Surgical Interest Group
2002-2003	President, Physicians for Human Rights
2001-2003	President, WVU School of Medicine (Class of 2005)
2000-2001	Graduate and Professional Health Advocate, Yale School of Music
1999-2000	Volunteer, Harmony Place; New Haven, CT
1998-1999	President, Carnegie Mellon Mortar Board Honor Society; Pittsburgh, PA
1998-1999	President, Sigma Alpha Iota Music Fraternity
1997-1998	Vice President, Sigma Alpha Iota Music Fraternity
1995-1999	Carnegie Mellon University Varsity Tennis Team; Co-Captain (1998-99, 1997-98)
1995-1997	Doctors of Carnegie, Vice President of Programming (1998-99), Vice President of Special Events (1997-98)
1998-1999	University Disciplinary Committee
1997-1998	Sexual Assault Advisor
1997-1998	Residence Hall Judicial Board
1997-1998	Volunteer, University of Pittsburgh Medical Center
1997-1998	Shadyside Hospital Preceptorship Program
1997-1998	Manager, Women in Sports House
1996-1997	Manager, Tennis House
1996-1997	Photographer, Tartan Yearbook
1995-1997	International Orientation Counselor
1995-1996	Regent Square Elementary, tutoring and mentoring program

Academic Honors and Professional Recognition:

2007	Research Recognition Award from the Cardiovascular Section of the American Physiological Society (APS)
2007	Van Liere Research Day Poster Presentation 1st Place Award, Level VI
2007	American Medical Association (AMA) Foundation Leadership Award (one of 15 medical students selected)
2006	Van Liere Research Day Poster Presentation 1st Place Award, Level II
2006	Sigma Xi Research Day 2nd Place Award, General Science Category
2006	Benjamin Zweifach Graduate Student Travel Award to the Annual Microcirculatory Society meeting
2006	Health Sciences Center Research and Graduate Education Travel Award
2004	Edward L. Reed Memorial Scholarship recipient for the 9th Biennial Symposium on Minorities, the Medically Underserved & Cancer
2004	Outstanding Student Honor Society (with Highest Honor)
2003	Recognized through the *Women in Medicine: "Strength through Diversity"* program to represent the WVU School of Medicine
2003	Gaydos Award, WVU School of Medicine, for the highest average in

Evidence Based Medicine and Health of the Public

2001 Awarded the Thomas Daniel Nyfenger Memorial Prize for a student who has "demonstrated the highest standard of excellence in woodwind playing at Yale University"

2000-2001 Emma Phipps White Memorial Scholarship, Yale University

1999 Chosen as one of twenty students in the United States to the 1999 All-USA College Academic First Team (USA Today newspaper)

1999 Concerto Competition winner; solo performance with the Carnegie Mellon Philharmonic Orchestra

1999 NCAA Division III ITA Scholar-Athlete award

1998 Best Physiologist of Semester, Carnegie Mellon University

1997 National Dean's List

1996 National Society of Collegiate Scholars, recipient of Merit Award

1996-1997 Lambda Sigma Honor Society

1995-1996 Horizons and Emerging Leaders programs at Carnegie Mellon

1995-1999 Dean's List eight consecutive semesters

1995-1999 Carnegie Mellon Music Scholarship

1995-1999 Andrew Carnegie Scholarship

Work Experience:

2005 Public Administration Internship at Morgantown Pulmonary Associates, a physician- owned and operated private corporation that focuses on respiratory illnesses. Posted insurance payments, completed and mailed insurance forms, aided with billing and collections, attempted to resolve outstanding insurance claims, helped with coding of procedures and diagnoses, contacted insurance companies regarding patient accounts, re-filed charts, copied medical records, served as interim medical transcriptionist, completed black lung and Department of Labor reports, typed staff meeting notes, generated Explanation of Benefits (EOBs) for secondary and tertiary claim submissions.

2000-2001 Visiting Artist Program, Pomperaug High School; Southbury, CT

1999-2000 Music Instructor, United Cerebral Palsy Association of Southern Connecticut; Waterford, CT

1999-2000 Waitress, Chart House; New Haven, CT

1999-2000 Administrative Assistant, Oral History of American Music and Norfolk Chamber Music Festival Offices, Yale University

1996-1997 Student Assistant, Graduate School of Industrial Administration, Carnegie Mellon University; Pittsburgh, PA

1995-1997 Lifeguard, Carnegie Mellon University pool

1995-1997 Office Assistant, Computer Science and Statistics departments, Carnegie Mellon University

Conferences:

2007 AMA National Advocacy Conference (NAC); Washington, D.C. Skills

learned include networking, creative problem solving, leadership, effective delivery and communication

2007 Federation of American Societies for Experimental Biology (FASEB); Washington, D.C. (abstract submission and poster presentation)

2007 American Educational Research Association (AERA) Annual Meeting; Chicago, IL (paper presentation)

2006 Paul Ambrose Political Leadership Institute, through the American Medical Student Association (AMSA); Washington, D.C. Skills learned include political strategizing, researching, public speaking, media relations and lobbying

2006 Southern Medical Association (SMA)- Southern Group on Educational Affairs (SGEA) Third Annual Conference on Graduate Medical Education; Galveston, TX (oral presentation with Dr. Scott Cottrell)

2006 Creating a Network of West Virginia Women in Science and Healthcare; Morgantown, WV (panelist)

2006 Federation of American Societies for Experimental Biology (FASEB); San Francisco, CA (abstract submission and poster presentation)

2006 Women's Health Policy Conference; Charleston, WV

2005 39th Annual Meeting of the West Virginia Political Science Association in conjunction with the American Society of Public Administration; Charleston, WV (panelist and moderator)

2004 19th Annual National MD/PhD Student Conference; Keystone, Colorado (abstract submission and poster presentation)

2004 Continuing Education Program on Quality and Accessibility of Health Care: Past, Present and Future. Part of the "100 Years: Health Professsions Education" at West Virginia University School of Medicine (panelist)

2004 38th Annual Meeting of the West Virginia Political Science Association in conjunction with the American Society of Public Administration, West Virginia Wesleyan College; Buckhannon, WV

2004 Intercultural Cancer Council (ICC) 9th Biennial Symposium on

Minorities, the Medically Underserved & Cancer; Washington, D.C.

2004 National Leadership Conference for Students in Healthcare; Washington, D.C.

Publications:

A. Theses

1. **J. Balch Samora.** Reliability and Sensitivity of Black-White Suicide Certification in the United States. M.P.H. Practicum, West Virginia University, 2004.

2. **J. Balch Samora.** Factors regulating arteriolar tone during microvascular growth. Ph.D. Dissertation, West Virginia University School of Medicine, 2007.

B. Abstracts

1. **Balch Samora J**, Frisbee JC, Boegehold, MA. Mechanisms of microvascular control during juvenile growth. 8th World Congress for Microcirculation, 2007, Milwaukee, WI.

2. **Balch Samora J**, Frisbee JC, Boegehold, MA. Hydrogen peroxide emerges as a regulator of arteriolar tone during microvascular network growth. XXXVII International Congress of Physiological Sciences, 2007, Washington, DC. The FASEB Journal;21(5): A494.

3. **Balch Samora J**, Frisbee JC, Boegehold, MA. Increased myogenic responsiveness of skeletal muscle arterioles with juvenile growth. XXXVII International Congress of Physiological Sciences, 2007, Washington, DC. The FASEB Journal;21(5): A494.

4. Frisbee JC, James ME, **Balch Samora J**, Goodwill AG, Chelladurai B, Basile DP. Low Vascular Nitric Oxide Bioavailability-Induced Skeletal Muscle Microvascular Rarefaction is not Associated with Increased Angiostatin Production. American Heart Association 7th Annual Conference on Arteriosclerosis, Thrombosis and Vascular Biology, 2006, Denver, CO.

5. **Balch Samora J**, Frisbee JC, Boegehold MA. Growth dependent changes in the endothelial factors regulating arteriolar tone. XXXVI International Congress of Physiological Sciences, 2006, San Francisco, CA. The FASEB Journal;20(4):A270.

6. Frisbee JC, James ME, Mikell C, **Balch Samora J**. Chronic nitric oxide synthase inhibition causes skeletal muscle microvessel rarefaction independent of increased mean arterial pressure. XXXVI International Congress of Physiological Sciences, 2006, San Francisco, CA. The FASEB Journal;20(4):A711.

7. Frisbee JC, Peterson J, James ME, **Balch Samora J**, Bryner R. Chronic exercise training improves vascular function and microvessel density in skeletal muscle of obese Zucker rats. XXXVI International Congress of Physiological Sciences, 2006, San Francisco, CA. The FASEB Journal;20(4):A813.

8. Rockett IRH, **Balch Samora J**, Coben JH, Smith GS. Suicide Rate Limits and the Black-White Paradox. American Public Health Association, 134th Annual Meeting, Injury Control and Emergency Health Services, 2006, Boston, MA.

9. Rockett IRH, **Balch Samora J,** Coben JH, Smith GS. The Black-White Suicide Paradox: Correcting Rates for Relative Underenumeration in Department of Health and Human Services, Centers for Disease Control and Prevention (CDC) INJURY AND VIOLENCE IN AMERICA: Meeting Challenges, Sharing Solutions, 2005: 83.

10. Marvar PJ, **Balch Samora J**, Boegehold MA. Reduced arteriolar responses to increased oxygen availability in rats fed high salt. XXXV International Congress of Physiological Sciences, 2005, San Diego, CA. The FASEB Journal;19(5):A1247.

11. **Balch Samora J**, Boegehold MA. Contribution of P450 4A enzymes to arteriolar oxygen responsiveness in skeletal muscle. 19th Annual National MD/PhD Student Conference, 2004, Keystone, Colorado.

C. Peer-Reviewed Manuscripts

1. **Balch Samora J**, Leslie N. The role of advanced practice clinicians in the availability of abortion services in U.S. *Journal of obstetric, gynecologic, and neonatal nursing,* 2007; 36(5): 471-476.

2. **Samora JB**, Frisbee JC, Boegehold MA. (in press). Hydrogen peroxide emerges as a regulator of arteriolar tone in skeletal muscle during juvenile growth. *Microcirculation,* 2007.

3. Outterson K, **Samora JB**, Keller-Cuda K. Will longer antimicrobial patents improve global public health? *Lancet Infectious Diseases,* 2007; 7(8): 559-566.

4. **Samora JB**, Frisbee JC, Boegehold MA. Growth-dependent changes in endothelial factors regulating arteriolar tone. *Am J Physiol Heart Circ Physiol*, 2007; 292(1): H207-214.

5. Cottrell S, **Samora JB**, Shumway J. An analysis of first-year medical student comments in a peer evaluation of professionalism. *Journal of the International Association of Medical Science Educators*, 2007; 17(1): 85-90.

6. Frisbee JC, **Samora JB**, Basile DP. Angiostatin does not contribute to skeletal muscle microvascular rarefaction with low nitric oxide bioavailability. *Microcirculation*, 2007; 14(2):145-153.

7. **Samora JB.** On the Wards: The Lessons of Loss. *The New Physician*, 2006; 55(6): 9-10.

8. Frisbee JC, **Samora JB**, Peterson J, Bryner R. Exercise training blunts microvascular rarefaction in the metabolic syndrome. *Am J Physiol Heart Circ Physiol*, 2006; 291(5): H2483-H92

9. Rockett IRH, **Samora JB**, Coben JH. The black-white suicide paradox: Possible effects of misclassification. *Social Science & Medicine*, 2006; 63(8): 2165-2175.

Current Research Interests:

Endothelium-dependent regulation of microvascular tone with growth and maturation.
Vascular smooth muscle control of blood flow during growth and maturation.
Antimicrobial resistance and health policy.
Methods to detect and eliminate iatrogenic injury.
Frequency and consequences of suicide misclassification.
Frequency and consequences of health disparities and methods to eliminate them.
Women's Health Issues.

Teaching:

Undergraduate

"Mechanisms of Body Function" Physiology 441, 300 undergraduate and graduate allied health students, Topics: Cardiac & Renal Physiology; West Virginia University School of Medicine, 2006.

"Elementary School Health Program" CHPR 301, 20 undergraduate students, Topics: Aspects of Personal Health; West Virginia University School of Medicine, 2007.

"Health of the Individual" CHPR 170, 40 undergraduate students, Topics: Mental Health, Reproductive Issues, DSM IV Paraphilias; WVU School of Medicine, 2007 and 2006.

Adjunct Professor, "History of Jazz," Class sizes roughly 20-30; Housatonic Community College; Bridgeport, CT, Fall 2000 & Spring 2001.

Bassoon Instructor for undergraduate students; Yale University New Haven, CT, 2000-2001.

Graduate/Medical

"Medical Pharmacology" PCOL 761, Small group facilitator; West Virginia University School of Medicine, 2007

"Issues in Men's Health" PUBH 619, 20 graduate students, Topics: Cardiovascular Disease, Diseases specific to men; West Virginia University School of Medicine, 2007.

"Issues in Women's Health" PUBH 621, 20 graduate students, Topics: Reproductive Issues; West Virginia University School of Medicine, 2007.

"Occupational Toxicology" PCOL 562, 15 graduate students, Topic: Developmental and Reproductive Toxicology; West Virginia University School of Medicine, 2006 and 2005.

"Human Form and Function" CCMD 730: Small group facilitator, Topic: Physiology; West Virginia University School of Medicine, 2005 and 2003.

Teaching Assistant for four graduate level courses at Yale University: "Hearing & Theory," "Music History Survey," "American Music History," "Modern Jazz: Bebop and Beyond" New Haven, CT, 2000-2001.

Seminars

Arteriolar function with growth and maturation: new insights. West Virginia University, Department of Physiology and Pharmacology, January, 2007.

Growth dependent changes in the endothelial factors regulating arteriolar tone. West Virginia University, Department of Physiology and Pharmacology, December, 2005.

High dietary salt and endothelial dysfunction: the role of tetrahydrobiopterin, eNOS, and superoxide. West Virginia University, Department of Physiology and Pharmacology, December, 2004.

Other

Music Affiliations:

Summer, 2001	Schleswig-Holstein Musik Festival, Germany
2000-2001	New World Symphony Orchestra, substitute; Miami, FL
2000-2001	Performed with Orchestra New England, New Britain Symphony, Sienna Orchestra of Waterbury
2001-2002	Invited as Principal Bassoon of the Daejeon Philharmonic Orchestra in Korea (declined)
1999-2000	Principal Bassoonist, Yale Philharmonic Orchestra
1999-2000	Principal Bassoonist, Yale Opera Orchestra

Summer, 2000	Music Academy of the West; Santa Barbara, CA
2000 & 1999	Sarasota Music Festival; Sarasota, FL
1996-2000	Jeunesses Musicales World Orchestra: Germany and Austria (Winter, 1999-2000); Poland, Germany, Canada, Switzerland, and the U.S. (Summer, 1999); Germany (Winter, 1998-99); Taiwan, Japan, Korea, and the Philippines (Summer, 1998); Germany and Poland (Winter 1996-97); Switzerland and Holland (Summer, 1996)
1995-1999	Principal Bassoonist, Carnegie Mellon Philharmonic Orchestra
1998 & 1996	Principal Bassoonist, Carnegie Mellon Opera Orchestra
1995-1996	Principal Bassoonist, Pittsburgh Youth Symphony Orchestra
1995	Principal Bassoonist, World Youth Symphony Orchestra; Interlochen, MI

Travel:

Fifty U.S. States, Argentina, Aruba, Australia, Austria, Bahamas, Barbados, Belgium, Belize, Brazil, Cambodia, Canada, China, Costa Rica, Curaçao, Czech Republic, Denmark, Dominican Republic, Egypt, England, Estonia, Finland, France, French Polynesia, Germany, Greece, Grenada, Holland, Honduras, Hong Kong, Indonesia, Ireland, Israel, Italy, Jamaica, Japan, Korea, Luxembourg, Macau, Malaysia, Martinique, Mexico, Norway, Panama, Paraguay, the Philippines, Poland, Puerto Rico, Russia, Scotland, Singapore, South Africa, Spain, St. Lucia, Sweden, Swaziland, Switzerland, Taiwan, Thailand, Turkey, Uruguay, Venezuela.